UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

In vitro evaluation of the toxicity of bismuth compounds

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Dissertação para obtenção do Grau de Mestre em
Ciências Biomédicas
(2º ciclo de estudos)

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Covilhã, outubro de 2016

Agradecimentos

Em primeiro lugar quero agradecer ao meu orientador, Professor Doutor Samuel Silvestre, por ter aceitado guiar-me neste percurso, por toda a disponibilidade, apoio e saberes transmitidos ao longo deste ano.

Quero agradecer também à Professora Adriana Santos, por toda a amabilidade, disponibilidade, apoio e conhecimento transmitidos.

Não podia deixar de agradecer também às minhas colegas de laboratório, Elisabete, Sandrina, Mariana, Mafalda, Sara e Vanessa pelos bons momentos, amizade e apoio.

Por fim, quero agradecer aos meus pais, à minha irmã e ao Ângelo Miguel por todo o carinho, pelo apoio e pelos sacrifícios que fizeram para me ajudar a estar onde estou hoje.

A todos, o meu sentido bem-haja!

Resumo alargado

O bismuto pertence ao grupo dos metais pesados, e demonstra um comportamento químico semelhante ao do arsénio e do antimónio, mas ao contrário destes elementos, o bismuto tem sido considerado relativamente não tóxico, uma vez que tem uma solubilidade em fluidos aquosos relativamente baixa. Contrastando com a extensiva informação existente sobre os demais elementos da tabela periódica, o bismuto tem, talvez, o aglomerado de informações menos desenvolvido, apesar de ser extensivamente usado na medicina. Os sais de bismuto são usados para tratar úlceras pépticas, dispepsia funcional e gastrite crónica. Apesar da falta de informação existente sobre o tema, é notório que a toxicidade por bismuto pode ser observada devido a ingestão abusiva ou mau uso aquando da ingestão em grandes quantidades ou por grandes períodos de tempo. Os efeitos tóxicos que têm vindo a ser reportados como causados por overdoses de compostos de bismuto incluem encefalopatias, nefropatias, osteoartropatias, gengivoestomatites e colites.

Como têm sido reportados na literatura alguns casos de toxicidade por bismuto, o objetivo deste projeto foi avaliar a toxicidade de alguns compostos de bismuto comumente usados na terapia, e como catalisadores de transformações orgânicas. Para isso, e utilizando o ensaio do brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT), os efeitos destes compostos na proliferação celular *in vitro*, foram avaliados. Este ensaio foi realizado com dois tempos de exposição aos compostos, 3 e 48 horas, para se avaliar se haveria toxicidade aguda e num tempo superior de exposição aos compostos, respetivamente. Para isso foram usadas linhas celulares representativas, incluindo neuronais (N27), intestinais (Caco-2), hepáticas (HepaRG) e mamárias (MCF-7) e fibroblastos da derme (NHDF). Nenhum dos dez compostos de bismuto estudados levou a uma redução significativa da proliferação celular após 3 horas de exposição, o que demonstra que os compostos estudados não provocam toxicidade aguda nas linhas celulares utilizadas. No entanto, após 48 horas de exposição aos compostos, foi observado que o triflato (III) de bismuto e o subnitrato de bismuto levaram a uma redução significativa da proliferação da linha celular neuronal (N27) e o subnitrato de bismuto leva também a uma redução da proliferação celular da linha celular intestinal (Caco-2) . Além deste ensaio, foram também executados o ensaio da citometria de fluxo usando iodeto de propídeo como marcador para as células mortas, uma vez que este composto intercala o ADN e emite fluorescência proporcional à quantidade de ADN da célula e o ensaio *do* 2',7' –dicllorofluorescina diacetato (DCFDA), que é um corante fluorogénico que mede espécies reativas de oxigénio; após a difusão para a célula o DCFDA é desacetilado pelas esterases celulares a um composto não fluorescente, que é posteriormente oxidado pelas espécies reativas de oxigénio a 2', 7' –diclorofluoresceina (DCF), que é um composto altamente fluorescente que pode ser detetado por espectroscopia de fluorescência. Estes ensaios foram realizados para se tentar ter alguma informação sobre os potenciais mecanismos de toxicidade mediados por estes compostos. Quando a produção de

espécies reativas de oxigénio aumenta e se ultrapassam as capacidades antioxidantes da célula, podem ocorrer danos macromoleculares principalmente no ADN, e em proteínas ou lípidos, o que pode levar à apoptose ou necrose. Com o ensaio do DCFDA foi possível medir indirectamente a formação de espécies reativas de oxigénio que os compostos triflato (III) de bismuto e subnitrato de bismuto provocam na linha celular N27. Neste ensaio foi observado que o composto triflato(III) de bismuto parece não ter um efeito na produção de espécies reativas de oxigénio, mas pelo contrario o composto subnitrato de bismuto parece ter algum efeito, numa exposição de 6 horas aos compostos. Com maior tempo de exposição ao composto subnitrato de bismuto, 24 horas, foi observado que o este composto na maior concentração testada leva à produção de espécies reativas de oxigénio, quase ao mesmo nível que o controlo positivo.

No ensaio de citometria de fluxo foi usada também a linha celular neuronal e também os compostos triflato (III) de bismuto e subnitrato de bismuto. Num estudo preliminar à citometria de fluxo, observou-se ao microscópioóptico a morfologia celular, tendo sido possível observar que, de facto, o número de células foi diminuído pela acção destes compostos, e que a morfologia das células neuronais, tanto pela ação do triflato (III) de bismuto, como do subnitrato de bismuto, ficou alterada após 24 horas de exposição. Os resultados da citometria de fluxo mostram que houve um aumento estatisticamente significativo da população de células mortas, com a exposição a estes compostos, apesar de não ser um aumento muito elevado. Principalmente com a exposição ao composto subnitrato de bismuto foi de notar um aumento estatisticamente significativo da população intermédia, que se suponha que sejam células a entrar em apoptose, detritos celulares, células auto-fluorescentes ou talvez composto precipitado. Assim, o ensaio da citometria de fluxo mostrou realmente alguma morte celular, estatisticamente significativa, mas não em grande dimensão. Estes resultados são congruentes com os resultados do ensaio do DCFDA, que detetou existir algum *stress* oxidativo, mas mais uma vez não em grande extensão.

Palavras-chave

Bismuto, citotoxicidade, cultura celular, viabilidade celular citometria de fluxo

Abstract

Bismuth belongs to the group of heavy metals and shows a similar chemical behavior to arsenic and antimony; but unlike these it has been regarded as relatively nontoxic mainly due to its relatively low solubility in aqueous fluids. In contrast to the comprehensive database of other stable elements in the periodic table, bismuth has, perhaps, the least well established data bank, although it has long been used in medicine. In fact, bismuth salts are used to treat peptic ulcers, functional dyspepsia and chronic gastritis. In spite of the low available information, it is known that bismuth toxicity may be observed due to excessive ingestion, or misuse when taken in large quantities and for a long period of time. The reported toxic effects caused by an overdose of bismuth compounds include encephalopathy, nephropathy, osteoarthropathy, gingivostomatitis and colitis.

As recently some clinical cases of bismuth toxicity have been described, our aim was to evaluate the toxicity of bismuth compounds commonly used in therapy and as catalysts in organic transformations. For this, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the *in vitro* cell proliferation effects of these compounds in representative cell lines such as neuronal (N27), intestinal (Caco-2), hepatic (HepaRG), breast (MCF-7) and in dermal fibroblasts (NHDF) were evaluated; it was observed that bismuth (III) trifluoromethanesulfonate and bismuth subnitrate led to a significant reduction of the proliferation of the neuronal cell line after 48h of exposition to the compounds. In addition, flow cytometry studies with propidium iodide staining and the 2',7' –dichlorofluorescein diacetate (DCFDA) assay, acellular reactive oxygen species detection assay) were performed intending to elucidate the potential mechanisms of cell death mediated by these compounds. The flow cytometry studies showed indeed some statistically significant cell death, but not in a great extent. These results are congruent with the DCFDA assay studies, which detected some oxidative stress, but again, not in a pronounced extent.

Keywords

Bismuth compounds, cytotoxicity, cell culture, cell viability, flow cytometry

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List of acronyms

BAL	2,3 – Dimercapto-1-propanol
BIPP	Bismuth Iodoform Paraffin paste
CBS	Colloidal Bismuth Subcitrate
CT	Computerized tomography
DCF	2',7'-dichlorofluorescein
DCFDA	*2',7'* -dichlorofluorescein diacetate
DMPS	2,3-Dimercapto-1-propanesulfonic acid
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediamine tetraacetic acid
EEG	Electroencephalography
FBS	Fetal Bovine Serum
MBP	Metal-binding protein
MRI	Magnetic Ressonance Imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffer saline
PI	Propidium Iodide
ROS	Reactive oxygen species
TBHP	Tert-butyl hydroperoxide

1 Introduction

1.1 Bismuth

It is thought that the name bismuth derives from the German word Weissmuth or Wismut, which means white substance. Bismuth has an atomic mass of 208.980 and is the heaviest stable element (83rd element of the periodic table, being the least abundant of the elements of the Group 15)[1]. Bismuth is sometimes classified as a semi-metal or metalloid, since it has the characteristics of a metal and possesses properties alike those of semiconductors and insulators[2]. Bismuth is a relatively rare element, with an abundance comparable to that of silver and mercury, although not quite as expensive since large amounts are recovered as a by-product of copper and tin refining[2]. The world production of Bismuth in 2015 was above 13000 metric tons and the main producers were China and Vietnam[3], as can be seen in figure 1.

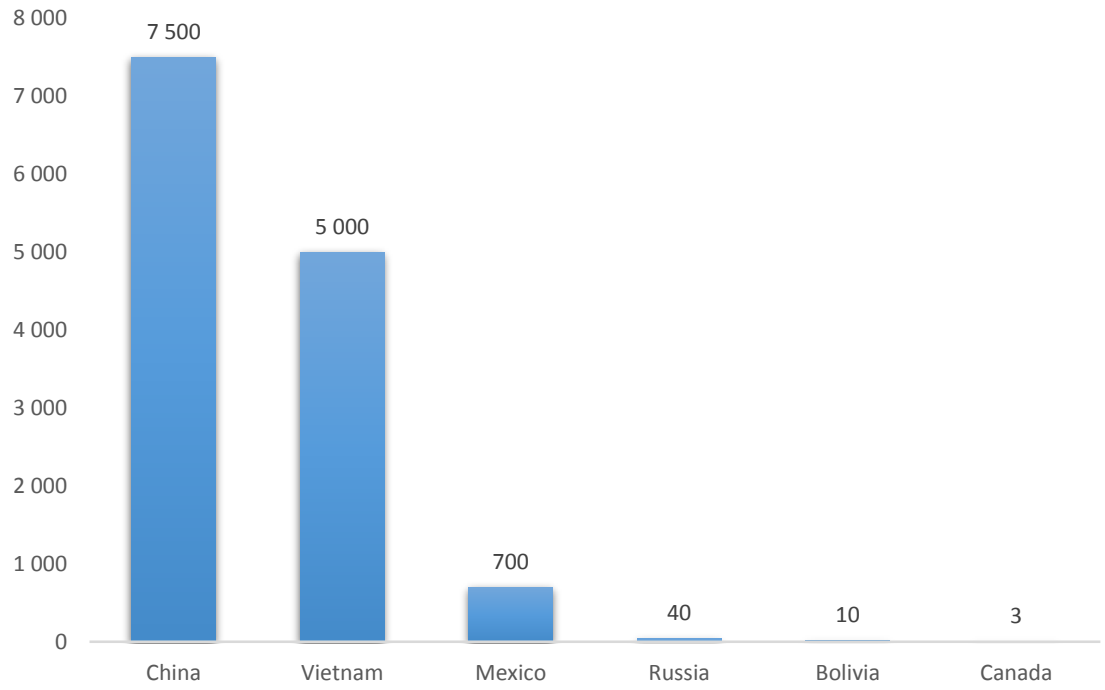


Figure 1 - Bismuth mine production in 2015

Estimated world bismuth reserves suggest that China is the country with more abundance of this element, followed by Vietnam, as shown in figure 2.

Bismuth is used in the most varied fields, and the most prominent use for this element is in low melting alloys and metallurgical additives, including electronic and thermoelectric applications[4]. Nonetheless bismuth is also used as a catalyst, in pharmaceuticals and industrial chemicals and as a pearlescent pigment in cosmetics.

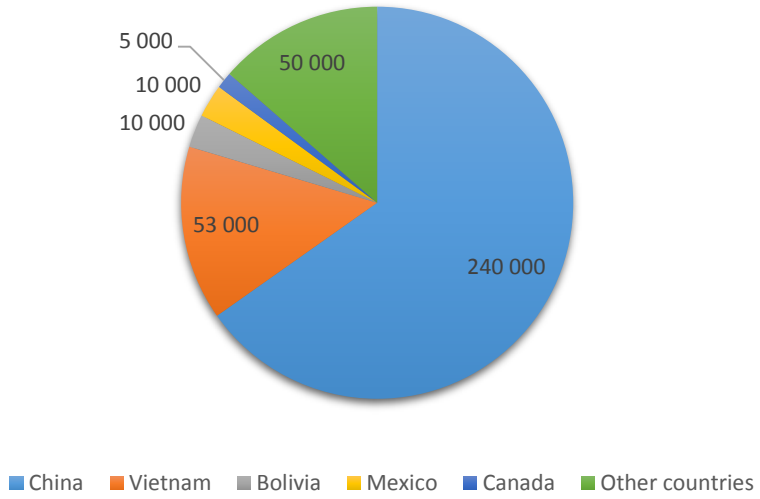


Figure 2 - Bismuth world reserves as of 2015 (metric tons)[5]

1.2 Bismuth environmental levels and exposure

Concentrations of bismuth in rural air range from 0.1 to 0.6ng/m^3 and in urban air this number can be between 1 and 66ng/m^3 [4]. The daily inhalation of bismuth is estimated to be less than 0.01-0.76μg [6].

In a recent review[7] it was reported that bismuth concentrations vary from 10 to 30 ng/L in seawater and from a few ng to a few μg/L in freshwater. As levels of bismuth in food are relatively low, 0.1 to 1μg/kg, the exposure to bismuth through water and food is likely to be minimal.

Bismuth levels in soil are roughly 1μg/kg, and in rocks like coal and sandstone, values range from 0.1 to 3μg/kg[8].

Exposure to bismuth can also occur through the use of cosmetics, as bismuth oxychloride is present in some cosmetics[9] mainly in those marketed as "mineral makeup", because it presents a distinct shimmery, pearlescent appearance and a fine white powder texture that adheres well to the skin. Therefore, mostly pharmaceuticals but also cosmetics are a source of more prolonged exposure to bismuth, but not to all population.

1.3 Bismuth in medicine

1.3.1 Bismuth compounds in medicine

Based in the gradually understood characteristics of this element, many bismuth compounds have been prepared and some have clinical and health applications. Bismuth salts have been used for over two centuries in the therapy of a large variety of clinical conditions including dyspepsia, diarrhea, syphilis, oral and upper respiratory tract infections, verrucae and warts[10].

The bismuth salts used over these two centuries have been diverse, including compounds such as bismuth subcitrate, bismuth subsalicylate, bismuth subgallate and others that are shown in table 1. The most relevant will be presented in the next subsections.

Table 1 - Bismuth compounds and its uses in medicine (adapted from [10])

Name	Therapeutic end
Bismuth aluminate	Antacid
Bismuth butylthiolaurate	Antisyphilitic
Bismuth-*D*-camphocarboxylic acid salt basic	Antisyphilitic
Bismuth chloride oxide	Antisyphilitic
Bismuth ethyl camphorate	Antisyphilitic
Bismuth iodide oxide	Antimicrobial
Bismuth iodosubgallate	Antimicrobial
Bismuth oxide	Astringent
Bismuth phosphate	Antacid
Bismuth potassium	Antisyphilitic
Bismuth sodium iodide	Antisyphilitic
Bismuth sodium tartrate	Antisyphilitic
Bismuth sodium thioglycollamate	Lupus erythematosus
Bismuth subcarbonate	Astringent
Bismuth subcitrate	Gastric and duodenal ulcers
Bismuth subgallate	Astringent/Antacid
Bismuth subnitrate	Antacid
Bismuth subsalicylate	Lupus erythematosus/ Antidiarrhoeal
Bismuth tannate	Astringent
Bismuth trobromophenate	Antimicrobial

1.3.1.1 Bismuth subcitrate

Bismuth subcitrate is a mineral used in the treatment of ulcers. Other names for bismuth subcitrate include colloidal bismuth subcitrate (CBS) and tripotassium dicitratobismuthate. According to a report by the European Medicine Agency[11] earlier in 2016, a drug containing this compound was authorized in Portugal (and other countries), with the name Pylera™, for the treatment of peptic ulcers with infections by *Helicobacter pylori*.

1.3.1.2 Bismuth subsalicylate

This compound was initially administered as an intramuscular injection for the control of syphilis[12,13]. This bismuth salt is nowadays used to treat heartburn, upset stomach, indigestion, nausea, diarrhea or symptoms associated with excesses in eating and drinking. It's used to decrease the number of bowel movements and make the stool firmer. It is thought that this

salt may limit the secretion on the digestive tract, reduce inflammation in the stomach and intestines, and inhibit the growth of certain bacteria and viruses that can cause intestinal tract diseases[14].

1.3.1.3 Bismuth subgallate

This compound is commonly used as an internal deodorant (for flatulence and stools).

1.3.1.4 Bismuth Iodoform Paraffin Paste

Bismuth Iodoform Paraffin Paste (BIPP) is an antiseptic agent that is widely used for packing wounds and cavities in the ear, nose and throat, and maxillofacial surgery, since it acts as a hemostatic agent, reduces wound colonization and promotes granulation tissue formation and wound repair[15].

Currently, the major medicinal use of bismuth compounds is focused in two fields: antimicrobial and anticancer [16].

Bismuth can interact with nucleotides and with amino acids in peptides, enzymes and other proteins, which are closely related to its uptake, accumulation, transport and excretion in the human body, and to their antimicrobial and anticancer activities[17,18].

There are currently 13 clinical trials with bismuth according to the Clinical Trial Registry[19], the majority of which concern its activity on *Helicobacter pylori*.

1.3.2 Bismuth as an antimicrobial and antiulcerous agent

For the past century bismuth compounds have been used in the treatment of various gastrointestinal disorders and microbial infections such as syphilis, colitis, wound infection, dyspepsia, diarrhea and peptic ulcers [20].

Bismuth subsalicylate, colloidal bismuth subcitrate and ranitidine bismuth citrate are used worldwide to treat various gastrointestinal diseases which are related to the infection of *Helicobacter pylori* [16]. *Helicobacter pylori* can prevent ulcers from healing, so bismuth compounds have also an anti-ulcer activity, due to the inhibition of the activity of this bacteria. In addition, bismuth cam precipitate within the ulcer crater, leading to the formation of a glycoprotein-bismuth complex, which acts as a protective coating and contributes to the healing of the lesion [18].

1.3.3 Bismuth as an anticancer agent

Biocoordination studies of bismuth compounds argument that the main target are non-DNA sites, which offers opportunities for new targeted approaches in the treatment of cancer [17,18,20-52]. Several synthetic bismuth molecules including organo- and inorgano- bismuth derivatives have been prepared by a number of research groups and evaluated in their *in vitro* cytotoxic

or antiproliferative activities against various cancer cell lines. The bismuth derivatives include bismuth dithiolates and dithiocarbamates, a water-soluble bismuth macrocycle complex, heterocyclic organobismuth derivatives, triarylbismuth bis (carboxylates), tris(2-(N,N-dimethylaminomethyl)phenyl) bismuth, and bismuth 8-quinolinethiolates [20-52]. Several compounds proved to have potent antiproliferative effects, which in some cases, are superior to those observed with cisplatin and other classical anticancer agents [20].

A known strategy for cancer treatment is the use of targeted radiation therapy, which is an approach mostly considered in inoperable tumors, tumors situated close to radiation sensitive organs, metastatic disease, and diseases such as leukemia and lymphoma. This therapy involves the use of carrier molecules, for example, antibodies (Ab) and peptides, specifically targeting cancer cells, and a selected radionuclide that should emit controlled doses of ionizing radiation to cancer cells without affecting healthy tissue surrounding them [53,54]. The most important variables that condition the selection of a specific radionuclide are its half-life and the existence of viable chemistry for this use or viable supply [20]. As ^{212}Bi and ^{213}Bi meet the baseline parameters that define reasonable use within this context, these radionuclides are probably the most studied α-emitters in this type of therapy. These radionuclides can be stably bound to several chelating agents that can be conjugated to monoclonal antibodies, peptides, or other vectors without significant safety measures or shielding required. The *in vivo* stable sequestration of *212*Bi and *213*Bi radionuclides is important to maximize their delivery of radiation to tumors and to minimize renal toxicity and other toxic effects. Several research groups have been developing ^{213}Bi-based systems to make rational improvements on chelation and/or radiolabeling chemistry, radionuclide delivery, targeting vectors, molecular targets and therapeutic strategies and performing *in vitro* and *in vivo* studies in several different cancer models [53,54].

Although ^{213}Bi compounds have high interest in cancer treatment, the development of radiotherapy involving this type of radionuclide has been limited by high costs, unresolved chemistry, and its limited availability. Furthermore, the *in vivo* stability and metabolism of these compounds is not well defined and radiologic side effects are still to be observed[20].

1.4 Bismuth's Pharmacokinetics

1.4.1 Absorption

The site of bismuth absorption in man has not yet been fully determined, but bismuth compounds are thought to be somewhat absorbed through the respiratory and intestinal tracts, depending on their solubility[4], but there are no quantitative data. The majority of ingested bismuth is not absorbed, but excreted mainly through the feces, and less than 1% of the administered dose is absorbed following oral dosing with bismuth subsalicylate[55], tripotassium dicitrato bismuthate[56] or ranitidine bismuth citrate[57].

Some animal studies suggest that the absorption takes place in the small bowel, although the rapid appearance of bismuth in blood after oral intake suggests bismuth can be absorbed in the stomach[58] Absorption through the skin is interesting, since bismuth compounds are used in cosmetics, but, once more, there are no quantitative data. An interesting study showed the rapid intake of bismuth into cells of the gastrointestinal tract and kidneys within hours of exposure, and some weeks later bismuth was found on a number of organ systems[59].
Bismuth is methylated by the bacterial flora in the gut, and excreted as bismuth sulfide, causing the blackening of the feces and sometimes also of the oral mucosa[60].

1.4.2 Distribution

It was demonstrated that after incubation of blood with radioactive bismuth citrate, 17% of the radioactivity was associated with erythrocytes and the remainder underwent non-specific binding to serum proteins[61]. A gel filtration study of human blood after incubation with bismuth subgallate showed an association of bismuth with the high molecular fraction (≥200.000 daltons) consisting of a α2-macroglobulin, IgM, β-lipoprotein and haptoglobulin[62].
Regarding bismuth distribution in the tissues, the highest concentration/g wet weight was always found in the kidney[58]. The retention time of bismuth in the kidney is longer than in any other organ. In other organs, 144 hours after intravenous injection of ^{206}bismuth citrate, 12% of the injected dose remained in the kidneys and 0.9% in the bone[61]. It was also demonstrated a retention in the kidneys of rabbits and dogs with soluble bismuth compounds[63].
Lee et al. [64] found a distribution pattern after administration of colloidal bismuth subcitrate to rats for 14 months. Bismuth concentrations were ordered from high to low in kidney (13.9μg/g wet weight), lung, spleen, liver, brain and muscle (0.13μg/g wet weight).
In patients who died from bismuth encephalopathy the highest concentrations were found in the thalamus and in the cerebral cortex, and additionally the concentration of bismuth in the grey matter was twice as high as the one found in white matter[65].
The knowledge that bismuth can be an effective inducer of metallothionein and that it can also bind this protein, has been applied as a protective measure against the nephrotoxicity of anticancer drugs such as cisplatin[66-68] and doxorubicin[69]. These groups observed in tumor-bearing mice and patients with renal cell carcinoma that orally administered bismuth was transported to normal tissues and not to cancerous tissues. Bismuth induction of metallothionein has been linked to an attenuation of the teratogenic effects of cadmium in mice[70] and the adverse effects of gamma irradiation on the bone marrows of mice[71].
The formation of trimethylbismuth in humans following ingestion of bismuth subcitrate was also reported[72], and a later study by others in this group showed that HepG2 cells were capable of methylating bismuth subcitrate and bismuth cysteine but not bismuth glutathione[73].

After a single subcutaneous injection with $BiCl_3$ in rats, it was demonstrated that bismuth binds to high molecular weight proteins in the kidney, but after repeated injections nearly all bismuth bound to a low molecular weight bismuth-metal-binding protein (Bi-MBP)[58]. Bismuth induces metal-binding protein (MBP) synthesis in the kidney.

The presence of intracellular particles after *in vitro* incubation of macrophages with colloidal bismuth subcitrate or bismuth subnitrate was demonstrated by light and electron microscopy[74,75]. Concentrations of colloidal bismuth subcitrate above 160μmol/L inhibit the *in vitro* migration of macrophages from spleen fragments; these effects indicate a possible intracellular cytotoxic effect of subcitrate particles on macrophages after phagocytosis[74].

After subcutaneous administration of high doses of bismuth subnitrate to rats, it was demonstrated that the mitochondria in the liver and proximal renal tubules underwent morphological changes, resembling swelling and distortion of the inner mitochondrial membrane[58].

1.4.3 Excretion

The majority of ingested bismuth is not absorbed, but excreted mainly through the feces, model values of bismuth elimination, with a daily intake of 20μg are a fecal elimination of 18μg, and a urinary excretion of 1.6μg[76].

The main elimination ways for absorbed bismuth is renal excretion, although biliary excretion may also be important[77].

The placenta is permeable to bismuth after intramuscular injection of potassium bismuth tartrate and sodium potassium tartro-bismuthate into pregnant rabbits and cats[78].

1.5 Bismuth toxicity

Despite the many beneficial qualities of bismuth, a variety of side effects, including neurological syndromes, have been recorded. The best documented case of its neurotoxicity was the outbreak of bismuth encephalopathy among numerous patients in France [79]. Bismuth accumulation has been shown in several cell types, including kidney cells [80], motor neurons [81], ganglion cells [82] and Leydig cells [83]. In all this cases, bismuth was found to be located in lysosomes, which play a vital role in heavy metal metabolism. Intralysosomal bismuth induces lysosomal rupture and decreased numbers of intact lysosomes [84].

Bismuth toxicity may develop due to excessive ingestion, or misuse when taken in large quantities and for a prolonged period of time [85].

The reported toxic effects caused by an overdose of bismuth compounds include encephalopathy, nephropathy, osteoarthropathy, gingivostomatitis, and colitis [86]. Bismuth poisoning mostly affects the kidney, liver and bladder. Chronic exposure to high levels of bismuth salts result in encephalopathy, whereas acute toxicity manifests as nephrotoxicity [87].

1.5.1 *In vitro* studies

Some studies have been made regarding the cytotoxicity of bismuth compounds, for example, Stoltenberg et al. [84] studied the bismuth uptake in the lysosomes of a histiocytic lymphoma cell line (J774 cells). These cells were exposed to several concentrations of bismuth citrate (5, 25, 100 and 200 μM) and different times of exposure (6, 12 and 24 hours) were evaluated. The authors concluded that cells exposed to concentrations higher than 5μM became less attached as a function of increasing exposure times. Damaged cells with disintegrated membranes were seen after an exposure of 12 or more hours to 100μM of bismuth citrate, and after only 6 hours of exposure to the concentration of 200μM [84].

The cellular uptake and the cytotoxic and genotoxic effects of bismuth compounds, namely monomethylbismuth, bismuth citrate and bismuth glutathione has been investigated by Von Recklinghausen *et al.* [60]. This group used HepG2 cells, human lymphocytes and human erythrocytes. Their results showed that the uptake of bismuth glutathione, was relatively low (<0,3%) in all these three cell types. On the other hand, the uptake of bismuth citrate by lymphocytes and erythrocytes was 2.6% and 6.5%, respectively, whereas the uptake of methyl bismuth was significantly higher (up to 23% by lymphocytes and 36% by erythrocytes).

In the Trypan Blue cytotoxicity test the most significant results were found with methyl bismuth[60]. In the hepatocytes, the cytotoxic effect was noteworthy after methyl bismuth treatment for 1 hour at concentrations above 350μM and after an exposure of 24h at concentrations above 130μM. In erythrocytes after 24 hours of exposure methyl bismuth was highly toxic at concentrations above 3.8μM (>50% cell death). In lymphocytes, methyl bismuth showed cytotoxicity only at high concentrations an exposure time (>430μM, 24h). After 24 hours of exposure, bismuth citrate showed cytotoxic effects in erythrocytes at concentrations above 113μM (48% cell death). This research group also established that after exposure of lymphocytes to methyl bismuth, chromosomal type aberrations occurred (single and double strand breaks). The results of Von Recklinghausen *et al.* [60] showed that the methylated bismuth compound was more membrane permeable and more cytotoxic than bismuth glutathione and bismuth citrate.

Dopp *et al.* [88] tested trimethylbismuth in Caco-2, CHO-9 and HepG2 cell lines. Their results showed that trimethylbismuth was cytotoxic in all three tested cell lines. Caco-2 cells were the most sensitive (LC_{50}: 110μmol/Lgv), followed by CHO-9 (LC_{50}: 128 μmol/Lgv) and HepG2 cells (LC_{50}: 194 μmol/Lgv).

The in vitro cytotoxicity of bismuth nanoparticles in HeLa and MG-63 cells was studied by Luo *et al.* [89] They concluded that HeLa cells are more prone to suffer cytotoxicity effects of various surface modified bismuth nanoparticles than MG-63 cells. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay they observed that the viabilities of HeLa cells decreased with the increase of the concentration of bismuth nanoparticles.

The *in vitro* neurotoxicity of bismuth ferrite nanoparticles on PC12 cells was evaluated by Song *et al.* [90] with a MTT assay. The results showed that cytotoxicity was dose-dependent, as cell vitality of the groups exposed for 3 hours, decreased from 95% to 73% with increasing exposure

concentrations from 10 to 200 µg/mL. The cell vitality further dropped to 65% when the concentration reached 500 µg/mL. The extent and mode of cell death has been assessed by the annexin V-FITC apoptosis detection kit, which evidenced that only a small percentage of cells undergo apoptosis (below 2%) and necrosis (below 10%) after exposure with bismuth ferrite nanoparticles in concentrations ranging from 50 to 200 µg/mL.

Using a human skin derived cell line, HaCaT keratinocytes, Gao *et al.* [91] showed that bismuth oxybromide induced a concentration-dependent loss of cell viability. Using the cytometric analysis of annexin-V/PI, the authors observed that bismuth oxybromide triggered mainly late apoptosis. Bismuth oxybromide caused disturbances in plasma membrane, and lead to a loss of membrane integrity and eventually cell death.

1.5.2 *In vivo* studies

Here we present a data collection of some *in vivo* studies concerning the toxic effects bismuth might produce.

Bismuth pellets gained popularity when the use of lead in shotgun pellets was forbidden, thus Pamphlett *et al.*[81] making use of the autometallographic (AMG) technique searched for bismuth that could be released from shotgun pellets that had been inserted into mice. The pellets were inserted into the peritoneal cavity, through an incision in the abdominal cavity of the mice. And this group evidenced that bismuth was present in the cerebrum (in neurons in the supraoptic, paraventricular, suprachiasmatic and arcuate nuclei), brain stem (neurons of the trochlear, oculomotor, mesencephalic trigeminal, abducens, facial and hypoglossal nuclei), spinal cord (cell bodies of large motor neurons), posterior root ganglia and in cell bodies of renal tubular cells, macrophages in the lung, and dendritic cells in the liver and spleen.

Stoltenberg *et al.* [83] investigated the detectable bismuth in testis of rats exposed to bismuth subnitrate using the AMG technique. This group found traces of bismuth in the interstitial tissue as well as in the seminiferous tubules; and an abundance of bismuth was also found in Leydig cells. The same research group [82] also aimed to determine whether bismuth is transported in motor and sensory axons by retrograde axonal transport. For this, bismuth subnitrate was intramuscularly injected in Wistar rats, and 3 days after the injection, this group detected bismuth in motor neurons of the ipsilateral spinal cord and in ganglion cells of the corresponding dorsal root ganglia. Bismuth was found to be located in lysosome-like organelles. To the best of our knowledge Stoltenberg *et al.* [82] were the first group to show that bismuth can access the nervous system by retrograde axonal transport.

The gastrointestinal absorption and systemic uptake of bismuth citrate or ranitidine bismuth citrate after oral exposure in female mice has been studied by Larsen *et al.* [59], again using the AMG technique. This group observed that bismuth is present and absorbed in gastrointestinal epithelial cells shortly after exposure as showed by bismuth staining in gastric, duodenal and epithelial cells. Using electron microscopy, the authors showed that bismuth was only seen in

lysosomes, and that higher bismuth concentrations increased the number of cells with signs of toxic degradation: cytoplasmic vacuolation and intracellular swelling.

Sano *et al*. [92] evaluated the toxicity of elemental bismuth in rats by an acute oral toxicity study and a 28-day repeated oral administration study. This group found no abnormal clinical signs in both of their studies. They predicted that the adverse toxic effects of bismuth as a simple metal substance would be low, when compared with the adverse effects of lead.

A 13-week intratracheal intermittent administration of bismuth study has also been reported by the same research group [93]. Low, medium and high dose levels of bismuth were tested (0.8, 4 and 20 mg/kg) and no abnormal clinical signs attributable to bismuth administration were found. However, hair loss was detected in 3 animals, in the medium and high dose levels, and suppression of body weight gain from day 29 forward, in the high dose level, but without statistical significance. A slight increase in erythrocyte count and mean cell hemoglobin concentration was also observed as well as pathological changes in the lungs and bronchial lymph nodes. Brown patches were observed in the lungs of animals of all dose levels. Black patches and lung collapses were detected in all animals from the two groups with higher concentrations of bismuth (4 and 20 mg/Kg). Enlargement of bronchial lymph nodes and a white patch in the liver was also observed in animals of all groups. These results show that bismuth inhalation can cause dose-dependent but not specific adverse effects.

1.5.3 Clinical evidences

Exposure to bismuth can cause renal failure associated with degeneration and necrosis of the epithelium of the renal proximal tubules, necrosis of the liver, reversible dysfunction of the nervous system, skin eruptions, and pigmentation of the gums and intestine [4].

In order to fully comprehend the scope of bismuth toxicity it is necessary to do a summary of the most recent clinical cases, namely those of overdoses with bismuth compounds.

A case was even reported of some side effects believed to be due to the use of bismuth in skin creams [94]. In fact, two patients presented intellectual impairment, memory loss, confusion, tremulousness, clumsiness, difficulty walking and myoclonic jerks. Bismuth was found in cerebral venous blood in both patients, and in the cerebrospinal fluid in one. It is proposed that bismuth can cross the blood/brain barrier and disturb oxidative cerebral metabolism [94].

1.5.3.1 Bismuth antiulcer agents

Probably the most notorious case of bismuth toxicity in man is the French outbreak of bismuth encephalopathy. In this context, Supino-Viterbo *et al*. [79] reported the case of 45 patients (8 male, 37 female) to which they had the opportunity of performing EEG studies, and study the clinical symptomatology in depth. All 45 patients had been treated with an insoluble bismuth salt (subnitrate) between 5 and 20 grams daily, over a period of 4 weeks going to 30 years. The blood bismuth levels (table 2, entry 1), taken on the same day as the EEG, ranged between 150

to 1600 µg/L (normal being less than 20µg/L). In urine samples the levels of bismuth were from 200 to 9600µg/L.

Hudson *et al*. [95] reported the case of a 27 year old man admitted 4 hours after an overdose of 100 tablets of De-Nol™ (12g of CBS), paracetamol and alcohol. The day after the patient was discharged as he felt normal. After 10 days the patient was admitted once more, complaining of anorexia, nausea, vomiting, general malaise, weakness of his legs, blurring of vision, thirst and poor urinary output. The patient showed no signs of encephalopathy. Some biochemical tests were made, and this group found that their patient had a blood bismuth level of 260µg/l and a urine bismuth level of 120µg/l (table 2, entry 2). An abdominal X-ray was performed, and it showed an opacification of the colon by ingested bismuth. An EEG showed non-specific slow wave changes over both hemispheres. The patient was diagnosed with renal failure and neurotoxicity induced by bismuth. The patient started hemodialysis and just five days later the renal function had returned to normal and the neurological signs were resolved.

A case of a 76 year old man that overdosed on 80 De-Nol™ tablets 4 hours prior to admission was reported by Taylor *et al*. [96] They performed an X-Ray that showed opacification of the colon; just as the case reported by Hudson *et al*. [95]. The authors detected 1600µg/l of bismuth in the blood (table 2, entry 3). The patient had already vomited in his home and in the emergency department and, for that reason, the gastric lavage was not performed. They noted that the patient was oliguric, and four hours later he began passing bloody stools. They started the patient on ranitidine, antacid and magnesium sulphate enemas. The patient was also dialyzed for 3 days, during which time he continued to pass bloody stools, and received a blood transfusion. He then developed acute abdominal pain with absent bowel sounds, but he was judged unfit for surgery and died 4 days later. Necropsy revealed a perforated duodenal ulcer and "pale kidneys" which proved to contain bismuth (11mg/g and 16mg/g) [96].

Other case reports have been described in medical literature, all evidencing that ingestion of high amounts of bismuth salts (> 5 grams) led to gastrointestinal, renal and neurological injury. For instance, Playford *et al*. [97] reported a case of a 68 year old man (table 2, entry 4) that for two months took double the recommended dose of De-Nol™ (864mg bismuth a day). On examination the patient evidenced cerebral dysfunction, incontinence, bilateral grasp reflexes, visual hallucinations and ataxia. An EEG demonstrated loss of alpha rhythm and diffuse slow waves consistent with a metabolic encephalopathy. The metal chelator 2-3 dimercapto-1-propane sulphonic acid (DMPS) was administered for 10 days and the patient´s EEG was normal after six weeks.

Tubular necrosis has been diagnosed in young adults after ingestion of toxic doses of bismuth compounds. Huwez *et al*. [98] reported the case of a 21 year-old man admitted 3 hours after ingesting 39 tablets of bismuth subcitrate (table 2, entry 5). The patient showed epigastric pain and an intravenous crystalloid infusion was prescribed, but over the next 2 days the urinary output fell and the renal function deteriorated. The renal biopsy revealed moderate acute tubular necrosis, although no bismuth was detected in the biopsy specimen. The patient was treated with intravenous frusemide, dopamine, mannitol and crystalloids.

A case of a 16 year old female that was complaining of nausea, vomiting and dizziness for 4 days and oliguria for 2 days has been described by Akpolat *et al*. [99]. One week before admission the patient had taken 10-15 tablets of tripotassium dicitrato bismuthane (table 2, entry 6). A renal biopsy was executed and revealed vacuolation, flattening, necrosis, and prominent regeneration in tubular epithelium. The final diagnosis was acute tubular necrosis.

A curious case of a 76-year old female was reported by Summers [100]; this woman's symptoms were misinterpreted as Alzheimer disease, but later were attributed to bismuth toxicity, since she had been ingesting a little more than 4 grams of bismuth daily (more informations on table 2, entry 7). The patient presented symptoms like confusion, poor appetite, disturbed sleep and muscle twitching. After treatment with penicillamine and Cognex™ the patient improved.

Accidental intoxication of a 2-year-old boy after taking 28 De-Nol™ tablets has been reported by Islek *et al*. [85]. Opacification of the intestine and colon was observed by abdominal X-Ray. On day 6 a control X-Ray exhibited no opacification. This group was only able to determine bismuth levels on day 10 (table 2, entry 8), and the levels were 739µg/l in the blood. The patient recovered and leaved the hospital on day 20 after admission. Blood bismuth levels were 96 µg/l and 12 µg/l on days 60 and 150, respectively.

Hruz *et al*. [101] reported a case of a 22-year-old woman (table 2, entry 9) who attempted suicide by taking 5,4g of colloidal bismuth subcitrate. Clinical examination showed a slight abdominal tenderness and pain over both renal flanks. The patient was started on intravenous treatment with the chelating agent DMPS. With the aim to eliminate bismuth, hemodialysis was started about 60 hours after bismuth ingestion.

Cengiz *et al*. [86] reported a case of a 16-year-old girl that came to the hospital complaining of nausea, vomiting and facial paresthesia. The authors were aware that 10 days earlier she attempted suicide by ingesting 60 De-Nol™ tablets. A physical examination showed periorbital and pretibial edema and facial paresthesia but no signs of encephalopathy. An abdominal ultrasonography revealed a slightly increased kidney. The serum bismuth levels 2 days after admission were 495µg/l (table 2, entry 10), and the patient was started on hemodialysis therapy It was also prescribe an oral treatment with a metal chelating agent (penicillamine). The patient left the hospital 16 days after admission, and in seven weeks her renal function returned to normal, and her serum bismuth levels had dropped to 260µg/l.

Reynolds *et al*. [102] reported a case of a 56-year-old woman who arrived to the hospital with several days of psychomotor retardation, decreased concentration, tremor of the hands, visual hallucinations and postural instability. The patient was being treated for irritable bowel syndrome, hypertension, hypothyroidism and depression. The patient later became delirious and somnolent and began to experience myoclonic jerks and hyperreflexia. An EEG on admission revealed moderate, but nonspecific, encephalopathy. Two months earlier the patient had begun taking bismuth subsalicylate to help control the diarrheal symptoms of her collagenous colitis, and she had been increasing her use of this medication over the past few weeks. The bismuth levels in the patient's blood was 397.3ng/mL (table 2, entry 11) and in the patient's

urine was 292.5ng/mL. The bismuth subsalicylate was held, and in the next two days the patient became more alert, had decreased myoclonus, and exhibited less muscular rigidity.

The case of a 21-year-old woman who was brought to the hospital 4 hours after taking 20 tablets of (CBS)in a suicide attempt (table 2, entry 12) was reported by Erden *et al*. [87] A gastric lavage was performed and the patient received intravenous fluid therapy. An abdominal ultrasonography demonstrated slightly increased echogenicity in the renal parenchyma. The patient became oliguric and then anuric. Blood chemistry and urine sediment showed signs of proximal tubular dysfunction with hypophosphatemia, hypouricemia, metabolic acidosis, and renal glycosuria despite normal plasma glucose concentration. The patient was started on a chelating agent, sodium-2,3-dimercapto-1-propanol, and hemodialysis. After 15 days the patient was discharged, but 8 weeks after discharge the patient's renal function test results remained high and the patient remained on hemodialysis for 1 year.

Akinci *et al*. [103] reported the case of a 16-year-old girl that came to the hospital 1 hour after taking 19 grams of bismuth subcitrate potassium (De-Nol™) in a suicidal attempt. She had no physical complaint and was conscious on admission. A gastric lavage was performed and the abdominal X-Ray showed opacity, so a whole bowel irrigation was performed. On the third day of admission the patient developed acute renal failure, metabolic acidosis and oliguria. The patient began hemodialysis following catheterization through the jugular vein. Bicarbonated dialysis were performed on the patient until the acute renal failure improved. Since the third day the patient suffered from sore throat, and on examination a bilateral tonsillar ulceration was found. On the 13th day the patient became polyuric, as a daily average of 15L of urine were excreted. On the 15th of admission the patient developed altered mental state, and the neurological examination revealed confusion, somnolence and cortical blindness. On the magnetic resonance imaging (MRI) scan, hyper-intense signal alterations were observed at the levels of bilateral parietal vertices of both cerebellar hemispheres. And intermittent rhythmic waves were detected in the frontal region on an encephalography examination. A neurologist diagnosed this patient with toxic metabolic encephalopathy. On the 20th day of admission the laboratory parameter of the patient began to normalize.

1.5.3.2 Bismuth iodoform paraffin paste

Bismuth iodoform paraffin paste (BIPP) contains two active ingredients, bismuth subnitrate and iodoform, and is used to pack cavities in ear, nose, throat, dental and neurosurgical practice. It is believed that BIPP acts as an antiseptic and astringent.

Sharma *et al*. [104] reported a case of a 57-year-old-woman who in May of 1991 got a basal cell carcinoma removed, and large areas of dura matter where exposed bilaterally with the intervening sagittal sinus. All were packed with BIPP. On July the patient became confused and agitated with intermittent bihemispheric signs, and eventually lapsed into a coma. A computed tomography (CT) scan of the brain showed diffuse cerebral oedema in both parieto-occipital lobes. In December the BIPP pack was finally removed, and the patient showed a progressive return to full alertness, rapport, cognition and coordinated bodily activity. In later December

a CT scan showed complete resolution of the cerebral oedema but also showed some patchy areas of high attenuation on the right parieto-occipital cortex subjacent to the exposed dura matter. Later a large BIPP pack was reapplied on order to obtain a clean granular bed for later grafting. After this reapplication of a BIPP pack the patient once again became confused, restless, dysarthric and insomnolent. On April of the next year the patient showed rapid deterioration in her conscious level, and became unresponsive. Only then the possibility of bismuth toxicity was considered, and the BIPP pack was removed. At this time blood bismuth concentration was 52ng/L (table 3, entry 1). The patient's conscious level improved, with the blood bismuth concentration falling to almost half by May. An MRI scan showed extensive cerebral oedema and hyperintense areas in the dura mater, central white matter, and periventricular ependymal lining. A more recent CT scan showed cerebral atrophy, but no evidence of tumor.

In another case[105] it was reported the situation of an 86-year-old women who was admitted to the hospital for a partial maxillectomy (as we can see in table 3, entry 2). The patient underwent the surgery with split skin grafting to the maxillary antrum, which was packed with BIPP. Five days after the surgery the patient was exhausted, lightheaded and unsteady. On day seven, the patient returned to the operating table for the replacement of the BIPP pack. The patient became increasingly aggressive, and on day 11 she was barely eating and having various fainting episodes. They did a CT brain scan, which was normal and electrolytes, liver function tests and full blood scan, were also normal. On day 14 the BIPP pack was removed, the patient was still confused and aggressive but 7 days after the removal of the BIPP pack the patient began to improve, and being cooperative; the patient was discharged 5 days later.

Three cases of allergic contact otitis externa due to BIPP was reported by Roest *et al.* [106]. All three cases were women who had their external auditory meatus and concha packed with BIPP-impregnated gauze following surgery. More information can be seen in table 3, entry 3.

Youngman *et al.* [107] described the case of an 81-year-old-man who suffered from epistaxis, and after 4 days of nasal packing, hemostasis wasn't achieved, and the patient underwent surgery. Two days after the surgery the patient's condition deteriorated, he became acutely confused, he also developed dysphagia, and was becoming incontinent. The surgeon had used nasal packing with BIPP when prolonged packing with Merocel™ failed to stop the epistaxis. The patient's serum bismuth level was 250µg/L (table 3, entry 4). This team stated that bismuth toxicity was the most likely cause of his temporary, but prolonged state of confusion.

A case of a 67-year-old man with a sacral chondroma that was surgically resected and after some troubles with the post-op the wound was irrigated with saline and packed with gauze soaked in BIPP was reported by Ovaska *et al.* [108] Five days after the packing with BIPP the patient became acutely confused, disorientated, delusional, and verbally aggressive to the staff. He was also suffering from abdominal discomfort, nausea and tremor, even though no cerebellar signs were present. By day 10 the patient's condition was deteriorating and bismuth toxicity was suspected as the patient had developed myoclonic jerks with intermittent episodes of drowsiness and worsening confusion. The blood and urine concentrations of bismuth were

determined and were 340µg/L and 2800µg/L (table 3, entry 5), respectively. The BIPP packing was removed and substituted with an alginate dressing. Due to the elevated bismuth concentration intravenous chelation therapy was initiated with DMPS. A total of 51 days of chelation therapy was administered. The patient's general condition improved significantly, and blood and urine bismuth levels declined.

Atwal *et al*. [109] reported 2 cases of reactions to BIPP packs (table 3, entry 6). In the first case a 59-year-old-man had a keratocystic odontogenic tumor packed with BIPP-impregnated gauze, followed by sequential replacement dressings. This patient became fatigued, confused, apathetic, forgetful, and he had spasms on his quadriceps. His blood bismuth concentration was 109.9nmol/L, so they removed the BIPP. After 18 months his blood bismuth concentration was 0.02nmol/L. In the second case [109] it was reported a 92-year-old-woman who got a BIPP pack placed after a right hemimaxillectomy. Nine days after the surgery she became progressively confused. Her blood bismuth concentration was 144.0nmol/L. The BIPP pack was removed and she gradually improved. About 4 months after the surgery and removal of the pack her blood bismuth concentration was 8.9nmol/L.

Table 2 - Reported cases of overdose of bismuth compounds

Entry	Gender	Age	Ingestion Form	Time from ingestion to hospitalization	Symptoms	Bismuth concentration before therapy	Bismuth concentration after therapy	X-Ray findings	EEG findings	MRI findings	Kidney biopsy	Therapy	Reference
1	M/F (45 patients)	24 to 80	5 to 20g of bismuth subnitrate daily	4 weeks to 30 years	Depression, anxiety, irritability, delusions, phobias, somnolence, sleep disorder, hallucinations, anorexia, motor incoordination, jerky movements	Blood – 150 to 1600µg/L Urine – 200 to 9600µg/L			Monomorphic waves at 3 to 5Hz; difuse beta rithm of low voltage				[79]
2	M	27	100 De-nol™ Tablets (12g colloidal bismuth subcitrate)	10 days	Anorexia, vomiting, nausea, weakness of the legs, blurring of vision, thirst, poor urinary output	Blood - 260µg/L Urine - 120µg/L Stools - 26.9 mg/g	96 days after ingestion: Blood - 8µg/g	Opacification of the colon	Non-specific slow wave changes to both hemispheres			Purgation (with magnesium sulphate); rehydration; hemodialysis	[95]
3	M	76	80 De-nol™ Tablets	4 hours	Confusion, epigastric tenderness	Blood - 1600µg/L		Opacification of the colon			Acute tubular necrosis	Ranitidine; antiacid, magnesium sulphate enemas Dialysis for 3 days	[96]
4	M	68	Twice the recommended dose of De-Nol™ (864mg daily) for 2 months		Cerebral dysfunction, incontinence, bilateral grasps reflexes, hallucinations and ataxia	Blood - 880µg/L Urine - 230µg/L			Loss of alpha rhythm and diffuse slow waves consistent with a metabolic encephalopathy			Heavy metal chelator 2-3 dimercapto-1 propane sulphonic acid (DMPS)	[97]

Table 2 - (Continued)

Entry	Gender	Age	Ingestion Form	Time from ingestion to hospitalization	Symptoms	Bismuth concentration before therapy	Bismuth concentration after therapy	X-Ray findings	EEG findings	MRI findings	Kidney biopsy	Therapy	Reference
5	M	21	39 tablets of bismuth subcitrate	3 hours	Epigastric pain	Blood - ~ 200µg/L Serum - ~ 1500µg/L	Blood - ~125 Serum- ~10				Acute tubular necrosis	Intravenous frusemide, dopamine, mannitol and crystalloids	[98]
6	F	16	10-15 tablets of tripotassium dicitrato bismuthate	1 week	Nausea, vomiting, dizziness and oliguria						**Acute tubular necrosis**	Hemodialysis, protein restriction, metoclopramide and aluminum hydroxide	[99]
7	F	76	Pepto-bismol™ (4.14mg daily for 7 years)		Confusion, poor appetite, disturbed sleep, muscle twitching	On day 6: Serum - 242µg/L	After 30 days: Serum - 90µg/L After 76 days: Serum - 14µg/L	Normal		Moderate atrophy, ventricular enlargement and ischemic white matter disease		Penicillamine, oral fluids, salt tablets, Cognex (Tacrine)	[100]
8	M	2	28 De-nol™ tablets (8.4g of colloidal bismuth subcitrate)	6 hours		On day 10: Blood - 739µg/L Urine - 693µg/L	Day 105: Blood - 12µg/L	Opacification of the intestine and colon	Normal			Gastric lavage, IV saline, mannitol, furosemide	[85]

Table 2 - (Continued)

Entry	Gender	Age	Ingestion Form	Time from ingestion to hospitalization	Symptoms	Bismuth concentration before therapy	Bismuth concentration after therapy	X-Ray findings	EEG findings	MRI findings	Kidney biopsy	Therapy	Reference
9	F	22	5.4g of colloidal bismuth subcitrate	2 hours		Day 3: Serum - 640µg/L	Day 11: Serum - 12µg/L			Enlarged and edematous kidneys with thinning of the cortical area		DMPS, hemodialysis, hemodiafiltrations	101
10	F	16	60 De-nol™ tablets	10 days	Nausea, vomiting and facial paresthesia	Day 12: Serum- 495µg/L	Day 64: Serum - 260µg/L		Normal			Hemodialysis, penicillamine	86
11	F	56	45mL (thrice per day) of bismuth subsalicylate (262mg/15mL)		Psychomotor retardation, decreased concentration, tremor of the hands, visual hallucinations and postural instability	Blood - 397.3ng/ml Urine - 292.5ng/ml			Moderate but nonspecific encephalopathy			Medication was held (bismuth subsalicylate)	102
12	F	21	20 colloidal bismuth subcitrate tablets (300mg of CBS)	4 hours				Normal	Normal			Gastric lavage, intravenous fluids, DMPS, hemodialysis	87
13	F	16	19 grams of De-nol™	1 hour			Hyper.intense signal alterations at the level of bilateral parietal vertices of both cerebellar hemispheres	Opacities in the left side of abdomen	Intermittent rhythmic waves in the frontal region	Hyper-intense signal alterations at the levels of bilateral parietal vertices of both cerebellar hemispheres			103

Table 3 - Reported cases of BIPP toxicity

Entry	Gender	Age	Operation/surgery	Symptoms after packing with BIPP	Bismuth levels	Observations	Reference
1	F	57	Removal of a basal cell carcinoma	Agitation, confusion, restlessness	52ng/L		[104]
2	F	86	Partial maxillectomy	Exhaustion, lightheadedness, poor appetite, tremor	Day 14 – 146nmol/L Day 22 – 81nmol/L		[105]
3	F	16	Myringoplasty	Mild erythema and swelling of the concha		Allergic contact otitis externa due to BIPP	[106]
	F	13	Myringoplasty			Allergic contact otitis externa due to BIPP	
	F	52	Mastoidectomy	Florid eczematous reaction		Allergic contact otitis externa due to BIPP	
4	M	81	Epistaxis treatment with BIPP packing	Acute confusion, dysphagia	250µg/L		[107]
5	M	67	Resection of a sacral chondroma	Acute confusion, disorientation, delusions, aggressive, abdominal discomfort, nausea, tremor	Blood – 240 µg/L Urine – 2800 µg/L		[108]
6	M	59	Marsupialisationa nd packing with BIPP of a keratocystic odontogenic tumour	Fatigue, confusion, apathy, forgetfulness and spasms in the quadriceps	Blood- 109.9nmol/L	After 18 months blood bismuth concentration was 0.02nmol/L	[109]
	F	92	Right hemimaxillectomy	Confusion	Blood – 144.0nmol/L	After 4 months blood bismuth concentration was 8.9nmol/L	

1.6 Treatment of bismuth poisoning

The contact with bismuth preparations should be stopped promptly in case of accidental or deliberate overdosing. According to the clinical cases reviewed above, elimination of bismuth from the body may be improved by hemodialysis, diuresis and the use of chelating agents, such as sodium-2,3-dimercapto-1-propanol (BAL), penicillamine and DMPS[87,97,101].

2 Objectives

Regarding all the information above, our main aim was to evaluate the toxicity of bismuth compounds commonly used in therapy and as catalysts in organic transformations. Seeing that so many clinical cases of side effects of bismuth compounds were reported, we set our goal to determine if, and to what extent, bismuth compounds are indeed toxic. For that purpose, the evaluation of the cytotoxicity of ten bismuth compounds by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, was performed. Flow cytometry studies using propidium iodide (PI) staining were also executed, to clarify the results of the previous study. The *2',7'* -dichlorofluorescein diacetate (DCFDA) study was also implemented in order to comprehend if bismuth compounds could perhaps lead to the production of reactive oxygen species (ROS).

Organic compounds, such as thiols, could enhance the solubility of bismuth compounds.[110] In addition, the toxicity of bismuth compounds has been partially associated to its coupling with endogenous thiols. For this reason, the Ellman's method was applied in order to realize if some bismuth compounds couple to thiols more than others, and in that fashion perceive if we could relate these results to the potential cytotoxicities of those compounds.

3 Materials and Methods

3.1 Compounds

In this project 10 bismuth compounds were studied:

- (B1) Bismuth(III) triflate, from Sigma-Aldrich
- (B2) Bismuth(III) nitrate, from Sigma-Aldrich
- (B3) Bismuth(III) chloride, from Fluka
- (B4) Bismuth oxychloride, from Acros Organics
- (B5) Bismuth(III) oxide, from Acros Organics
- (B6) Bismuth(III) subnitrate, from Sigma-Aldrich
- (B7) Bismuth(III) subsalicylate, from Sigma-Aldrich
- (B8) Bismuth(III) citrate, from Sigma-Aldrich
- (B9) Bismuth(III) gallate basic hydrate, from Sigma-Aldrich
- (B10) Bismuth carbonate oxide, from Alfa Aesar

The solutions of the compounds were all freshly prepared, for each single experiment. All the compounds were diluted in mili-Q water to obtain a concentration of 10mM. This solution was then sonicated 30 to 45 minutes at a temperature of 40 to 60ºC, to favor the solubility of the compound[91]. From this mother-solution appropriate dilutions of the compounds, in the different concentrations needed, were prepared in complete culture medium, before each experience. These prepared solutions were, once again, sonicated for 10 to 15 minutes at approximately 37ºC, so that then they could be applied to the cells.

3.2 Experimental Procedures

3.2.1 Biological Evaluation

3.2.1.1 Cell Cultures

In this study the cell cultures used were epithelial cells from a human colorectal adenocarcinoma (Caco-2) from passages 69 to 70, epithelial cells from a mammary gland adenocarcinoma (MCF-7) from passages 22 to 23, cells from a hepatoma of a female patient with cirrhosis subsequent to hepatitis C virus infection (HepaRG), kindly provided by Professor Gilberto Alves, from passages 15 to 18, non-carcinogenic human dermal fibroblasts (NHDF) from passages 11 to 13 as well as rat dopaminergic neural cells (N27) from passages 4 to 14, all acquired from the American Type Culture Collection (ATCC; Manassas, VA, USA). The chemicals (of analytical grade), assay reagents, culture mediums and supplements were all obtained from Sigma-Aldrich.

All the cell lines were cultured in 75cm^3 or 175cm^3 culture flasks, and maintained at 37ºC in a humidified atmosphere incubator with 5% CO2.

Caco-2 cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 1% antibiotic mixture of 10,000 U/mL penicillin G, 100mg/mL streptomycin.

MCF-7 cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic/antimycotic (10,000 U/mL penicillin G, 100 mg/mL streptomycin and 25 µg/mL anfothericin B).

HepaRG cells were cultured in Williams' medium E, supplemented with 10% FBS, 500µL/L insulin, 0.08mM hydrocortisone and 1% antibiotic mixture of 10,000 U/mL penicillin G, 100mg/mL streptomycin.

NHDF cells were cultured in RPMI 1640 medium supplemented with 10%FBS, 2mM *L*-glutamine, 10mM HEPES, 1mM sodium pyruvate and 1% antibiotic/antimycotic (10,000 U/mL penicillin G, 100 mg/mL streptomycin and 25µg/mL anfothericin B.

N27 cells were cultured in RPMI 1640 medium with 10% FBS and 1% of antibiotic mixture of 10,000 U/mL penicillin G, 100mg/mL streptomycin.

For all cell types, the medium was renewed every 2-3 days until the cells reached approximately 80-90% of confluence, at that moment the cells were detached from the culture flask by gentle trypsinization, 125mg/L trypsin in phosphate buffer solution (PBS) and 0.02g/L ethylenediamine tetraacetic acid (EDTA), and before the experiments, viable cells were counted with the trypan-blue exclusion assay and adequately diluted in complete culture medium.

3.2.1.2 MTT cell proliferation assay

After the process of trypsinization, and cell counting, 96-well plates (Nunc, Apogent, Denmark) were seeded with a cellular suspension with density of 2×10^4 cells/mL, with 100µL per well, and left to adhere for 48 hours. After that, the medium was replaced by the solutions of the compounds for the concentration-response studies (0.01, 0.1, 1, 10, 100 µM) in the appropriate medium for 3 and 48 hours. Untreated cells, to which the initial medium was replaced by fresh medium, were used as negative controls. Each experiment was performed in quintuplicate and independently repeated at least two times.

The *in vitro* antiproliferative effects were evaluated by the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, by measuring the extent of the MTT reduction. After the incubation periods (3 and 48-hours) the medium was removed, 100µL per well of phosphate buffer saline (NaCl 137mM; KCl 2.7mM, Na_2HPO_4 10mM and KH_2PO_4 1.8mM in distilled water and pH adjusted to 7.4) were used to wash the cells and then 100µL of the MTT solution (5mg/mL), prepared in serum free medium, were added to each well, followed by a 4-hour incubation at 37ºC. After the incubation period, the MTT containing medium was carefully removed and the formazan crystals were dissolved in DMSO. The absorbance was measured at 570nm using Bio-Rad xMark™ microplate spectrophotometer. Cell viability values were expressed as relative percentages of the absorbance in comparison with the respective controls.

3.2.1.3 DCFDA assay

After the process of tripsinization, and cell counting, in a 96-well black plate (Greiner) with a clear bottom, 100µL per well were seeded with a cellular suspension of 2.5×10^4 cells/mL, and left to adhere for approximately 24 hours.

In one of the experiments, after the adherence time (24 hours), each well was washed with PBS and then the cells were stained with PBS containing 20µM of DCFDA (diluted from a stock solution in dimethyl sulfoxide)[111]; unstained cells serve as negative controls. The microplate was then incubated for 45 minutes in the dark at 37ºC. After the incubation period, the DCFDA solution was removed and the wells were washed once more with PBS. After that the previously diluted compounds of interest were added to the plates, and left to incubate for 6 hours. For this specific assay the compounds were diluted in PBS supplemented with 2% serum, instead of culture medium. *Tert*-butyl hydroperoxide (TBHP) was also diluted to a concentration of 50µM, as a positive control. After the incubation period, the pate was read in a fluorescence plate reader (Spectra Max Gemini EM, Molecular devices), in the presence of the compounds, with excitation wavelength at 485nm and emission wavelength at 535nm.

As DCFDA is not stable for more than 6 hours, in order to test the compounds for a longer period (24 hours), some adjustments were made. The compounds were diluted in complete culture medium, and wells with medium and without cells served as blanks. After the 24-hour adherence period the cells were treated with the compound of interest (100µL per well) and the positive control compound (TBHP), and left to incubate for another 24 hours. 1 hour prior to completion of the treatment, DCFDA was diluted at two times the desired concentration (20 µM) in culture medium, and overlaid on top of the treated cells, 100µL per well. The plate was then incubated for 30 to 45 minutes at 37ºC. At the end of the incubation period the plate was read in the fluorescence plate reader, without washing, in the presence of compounds with excitation wavelength at 485nm and emission wavelength at 535nm.

3.2.1.4 Flow cytometry

With the flow cytometry technique, the cell viability was analyzed after staining the dead cells with propidium iodide (PI). The cells were seeded in a 12-well culture plate, with a density of 3×10^4cells/mL, at 1mL per well. After 24 hours the cells were treated with compounds B1 and B6, at a concentration of 50µM, for another 24 hours. Untreated cells were used as controls. At the end of the incubation period and before the flow cytometry assay, the effects of the compounds on the cell's morphology was performed through an optic microscope (Olympus CKX41) coupled to a digital camera (Olympus SP-500UZ) and several photographs were taken (Zoom:100x). After that, the supernatant of each well was collected and pooled with the cells harvested by tripsinization. The resulting cell suspension was kept on ice and pelleted by centrifugation, the pellet was then resuspended in 400µL of complete medium.

Subsequently, 397.5µL of the cell suspension were transferred to a FACS tube containing 2.5 µL of a solution of propidium iodide (Invitrogen) at 1mg/mL, and left to act for at least 5 minutes

protected from the light. A minimum of 20 000 events were acquired using a FACSCalibur flow cytometer using FSC, SSC and FL3 channels. Both the acquisition and the analysis were performed using the software CellQuest™Pro. In order to analyze the results a region (R4) was created (not shown) on the SSC/FSC contour plots to exclude part of the debris. At the FSC/FL3 contour plot, gated on R4, three additional regions were created: R1, concerning viable cells; R2, representative of dead cells; and last R3, which represents an intermediate population. The percentage of events was calculated relating the number of events in each region with the total number of events on R1, R2 and R3.

3.2.2 Thiols Quantification

With the Ellman's method we tried to determine if some bismuth compounds coupled to thiols from cysteine, more than others.

First a calibration curve of cysteine was determined. Two concentrations of each compound were prepared from the mother-solution (10mM), at 82 and 123µM, in the reaction buffer, 0.1M sodium phosphate, ph 8.0, containing 1mM EDTA.

Then, in each well of a clear 96-well microplate (Greiner bio-one), 100µL of reaction buffer plus 2µL of Ellman's Reagent solution (4mg/mL of reaction buffer) were placed, and 10µL of each compound solution and 10 µL of the chosen cysteine solution (100 µM) were also placed in 3 wells each. The plate was mixed and incubated at room temperature for 15minutes. After the incubation time the absorbance was read at 405nm using the Bio-Rad xMark™ microplate spectrophotometer.

3.2.3 Statistics

In the MTT assays each experiment was performed in quintuplicate and independently repeated at least two times. The flow cytometry studies were performed in triplicate and independently repeated at least two times. The results of those assays were expressed as average±standard deviation. The *t*-Student test was applied to determine statistical significance ($p<0.05$) in the cell proliferation results. These calculations were performed using the Microsoft Excell 2010 software. The IC_{50} values were calculated from the concentration-response curve by a sigmoid fitting, with a confidence interval of 95%. The cell viability in the flow cytometry studies, in regions R1, R2 and R3 was calculated with a confidence interval of 95%.

In the DCFDA assay each experiment was performed in quadruplicate and independently repeated at least two times. The results of this assay were expressed as average±standard deviation. Again, the *t*-Student test was applied to determine statistical significance ($p<0.05$).

4 Results and discussion

4.1 MTT assay

The anti-proliferative evaluation of the compounds' cytotoxicity was executed through the MTT assay, involving cell culture, the subsequent cell treatment with the compounds and the MTT assay, and the posterior analysis. The biological evaluation through the MTT assay is a reliable procedure, being widely used[112].

The MTT molecule is reduced to succinate dehydrogenase in viable cells, resulting in formazan, a purple water insoluble derivative. For this reason, the biosynthesis of formazan is considered proportional to the cell number, therefore the more intense the purple color is, the greater the number of living cells. The intensity of the purple color is then quantified spectrophotometrically[113].

The cell types included in the study are widely used, and are specifically pertinent in this study since, as stated before, bismuth is thought to accumulate namely in the intestine and liver[59] and can cross the blood brain barrier[94].

All compounds were tested via concentration-response curve studies, done with the five cell types mentioned, in two time frames, 3 and 48 hours of exposure to the compounds.

None of the compounds tested appeared to cause a decrease in cellular viability after 3 hours of exposure, which demonstrated that the compounds tested do not induce acute toxicity in the cells used. On the contrary, with a 48-hour exposure to the compounds, some effects are noticeable. It is noteworthy that N27 cells are the most affected by the compounds, in particular compound B1 and B6. In the Caco-2 cell line only the compound B6 had a limited effect. In the HepaRG cell line some compounds do present a IC_{50}, compound B6 and compound B9, but a high one. In the MCF-7 cell line, none of the compounds was cytotoxic. At last, in the NHDF cell line, compound B4 had a lower IC_{50}. A group[60] showed that bismuth citrate did not induce cytotoxic effects on hepatocytes, which can be congruent with our studies since for compound 8 (bismuth citrate) we found no IC_{50}.

Dopp *et al*.[88] found that Caco-2 cells were more sensitive to methylated bismuth than hepatoma or ovary cells. In addition, Song *et al*.[90] found that bismuth ferrite was toxic only at an exposure of 3 hours, prolonged that time, they concluded that the cells could recover to a certain extent with incubation time. Another study[91] reported that bismuth oxybromide induced a loss of cell viability in human keratinocyte cells.

Since compounds B1 and B6 were the most promising, in the N27 cell line, the following studies were made with these compounds and this cell line.

Table 4 - Half maximal proliferation inhibitory concentration activity (IC_{50}) values (μM) - 95% confidence intervals, for an exposure to the compounds of 48 hours. ND – Not Defined

Compound	Caco-2		N27		HepaRG		MCF-7		NHDF	
	IC50	R^2	IC50	R^2	IC50	R^2	IC50	R^2	IC50	R^2
B1	>100	-	14.22	0.99	>100	-	>100	-	ND	
B2	>100	-	21.06	0.99	>100	-	>100	-	ND	
B3	>100	-	>100	-	>100	-	>100	-	88.15	0.87
B4	>100	-	>100	-	>100	-	>100	-	46.2	0.87
B5	>100	-	>100	-	ND		>100	-	>100	-
B6	50.84	0.93	14.01	0.98	89.05	0.99	>100	-	>100	-
B7	>100	-	>100	-	ND		ND		>100	-
B8	>100	-	32.69	0.96	>100	-	>100	-	72.05	0.95
B9	>100	-	>100	-	83.19	0.86	ND		>100	-
B10	>100	-	>100	-	>100	-	>100	-	>100	-

4.2 DCFDA assay

The DCFDA – cellular reactive oxygen species detection assay uses the cell permeant reagent DCFDA, a fluorogenic dye that measures hydroxyl, peroxyl and other ROS presence within the cell[114]. After diffusion into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF)[115]. This compound in highly fluorescent, and can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495nm and 529nm, respectively[114].

When ROS production increases and overwhelms the cellular antioxidant capacity, it can induce macromolecular damage, by reacting with DNA proteins and lipids, which can lead to apoptosis or necrosis[116]. The increase of ROS production can also disrupt thiol redox circuits, which can lead to aberrant cell signaling and dysfunctional redox control[117].

In graphic 3, it can be seen the ROS detection on N27 cells after treatment during 6 hours to the compounds B1 and B2 and to TBHP, the positive control. It can be seen that compound B1 appears to not have a significant effect in ROS production, unlike compound B6 which appears to have a low but statistically significant increase in its lower concentration.

In graphic 4, the response of N27 cells to an exposure of 24 hours to compound B6and TBHP (positive control) is presented, and it can be observed that this compound lead to a significant increase in the production of ROS in its highest concentration, which almost matches the achieved with TBHP. Gao *et al*. demonstrated the generation of ROS in human keratinocyte cells for bismuth oxybromide. The production of ROS by compound B6 could be related to its effects on cellular viability, considering that elevated ROS production could lead to apoptosis or necrosis.

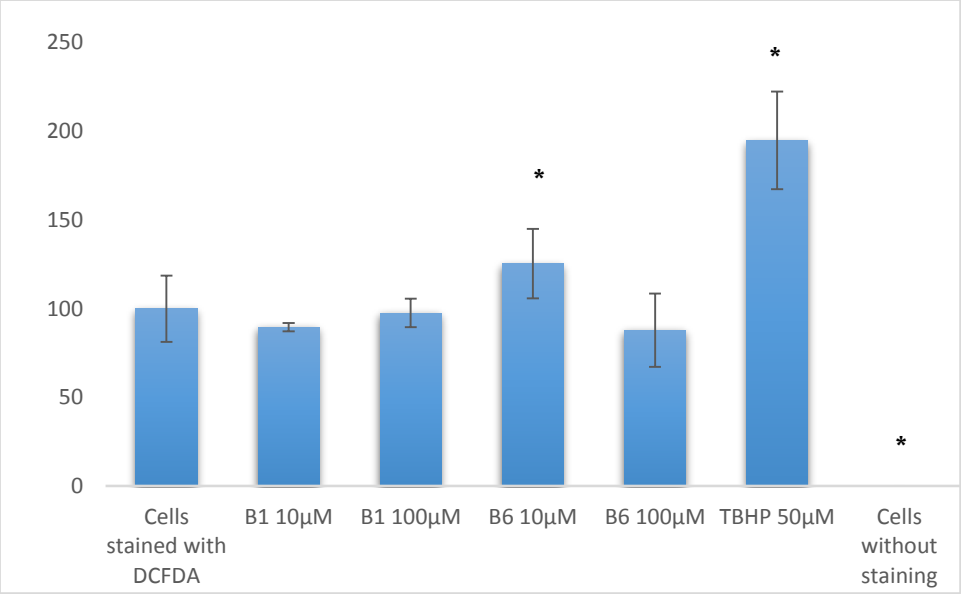


Figure 3 - DCFDA assay with N27 cells, exposure to the compounds of 6 hours. Results expressed in mean± standard deviation). TBHP was used as positive control. * p<0.05 in relation to a negative control (t-student test).

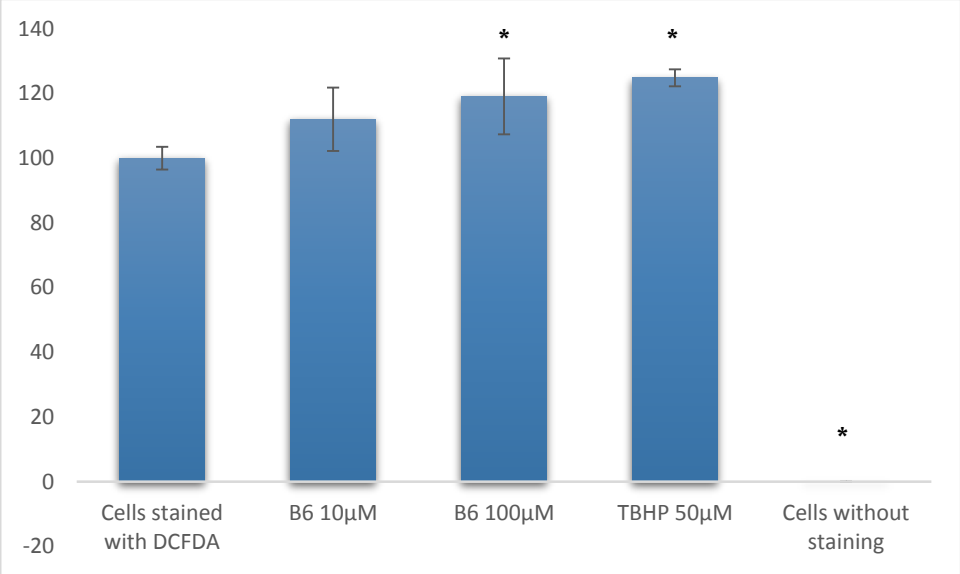


Figure 4 - DCFDA assay with N27 cells, exposure to the compounds of 24 hours. Results expressed in mean± standard deviation). TBHP was used as positive control * p<0.05 in relation to a negative control (t-student test).

4.3 Flow cytometry

The effects of compounds B1 and B6 were chosen to be further studied by flow cytometry studies, as they presented pronounced effects on cell proliferation, particularly in the N27 cell line. To analyze cell viability after treatment with these two compounds, flow cytometry assays were performed on N27 cells. Propidium iodide (PI) was applied to identify dead cells, since it is able to permeate comprised cell membranes. This compound intercalates DNA and emits fluorescence proportional to the DNA content of the cell[118].

Before flow cytometry analysis, to study cell morphology at 24 hours of exposure to the compounds, photographs were taken (Figure5) . In relation to the control, it is quite evident that both compounds induced a diminution of the cell number. Additionally, the cell morphology is clearly altered. In the control the cells are all in their differentiated form, elongated. With both treatments with compounds B1 and B6, the morphology of the cells is evidently changed. With the treatment with compound B1 some cells remain "normal" but numerous cells have an altered, rounded, morphology. With the treatment with compound B6 a few cells remain with a normal appearance, but numerous cells have a clearly altered morphology, it is also noticeable some precipitated compound, which is explained by the low solubility of bismuth compounds in aqueous solutions.

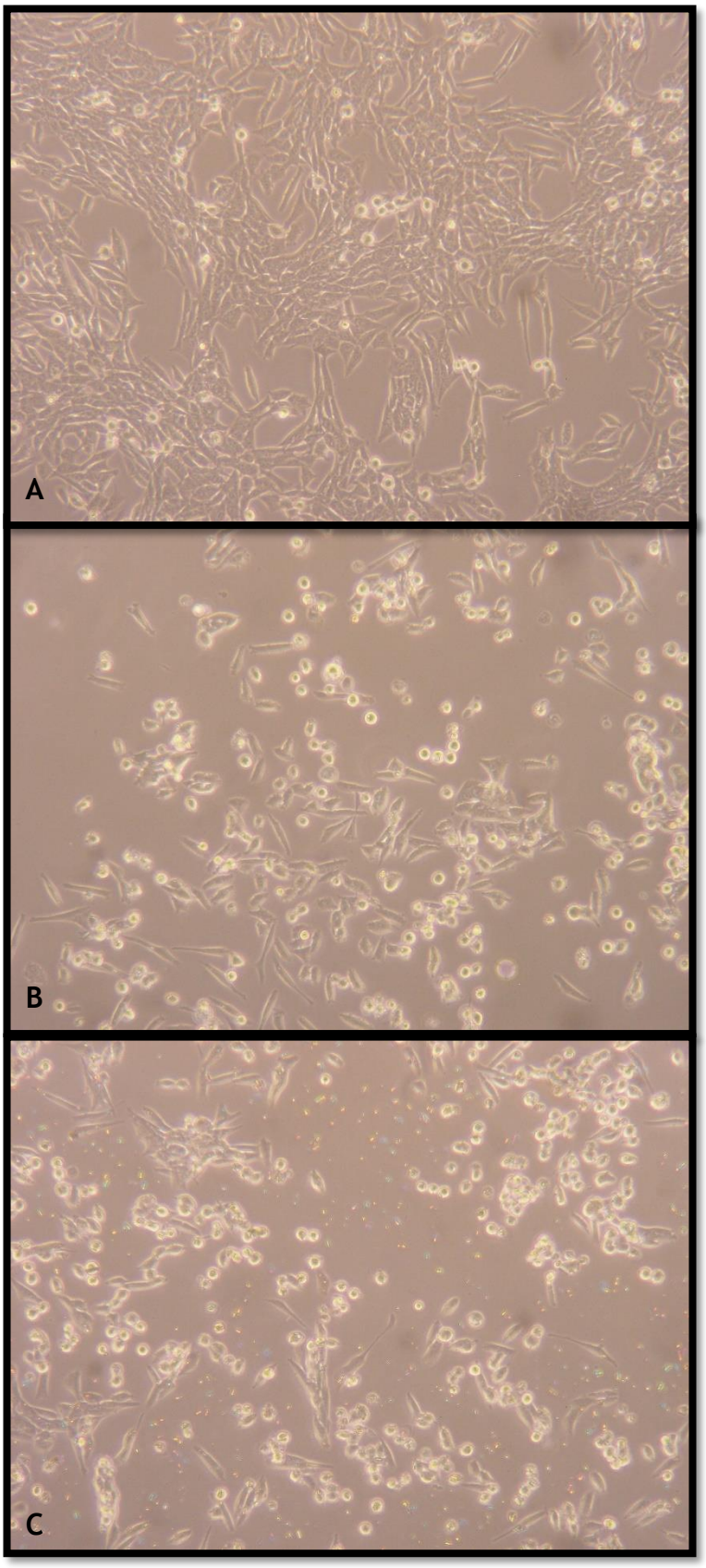


Figure 5 - Morphology of N27 cells after 24 hours of treatment; A - cells not treated (control); B - cells treated with compound B1; C - cells treated with compound B6. Zoom: 100x

The results of the flow cytometry assay were analyzed. Representative contour plots of FSC intensity versus PI intensity, a view of size of events against the fluorescence at 24h, are shown in figure 6. The events are divided in three different regions, R1, R2 and R3. In the R1 population, in general, the events have higher size and negative fluorescence, being considered viable cells. Usually the R2 population presents lower size and a positive fluorescence. R3 population has intermediate size and fluorescence, being considered early apoptotic or oncotic cells, cell debris, or autoflourescent cells. This area cannot be discarded, since it has a considerable number of events. Events out of these three main areas were not considered in the analysis.

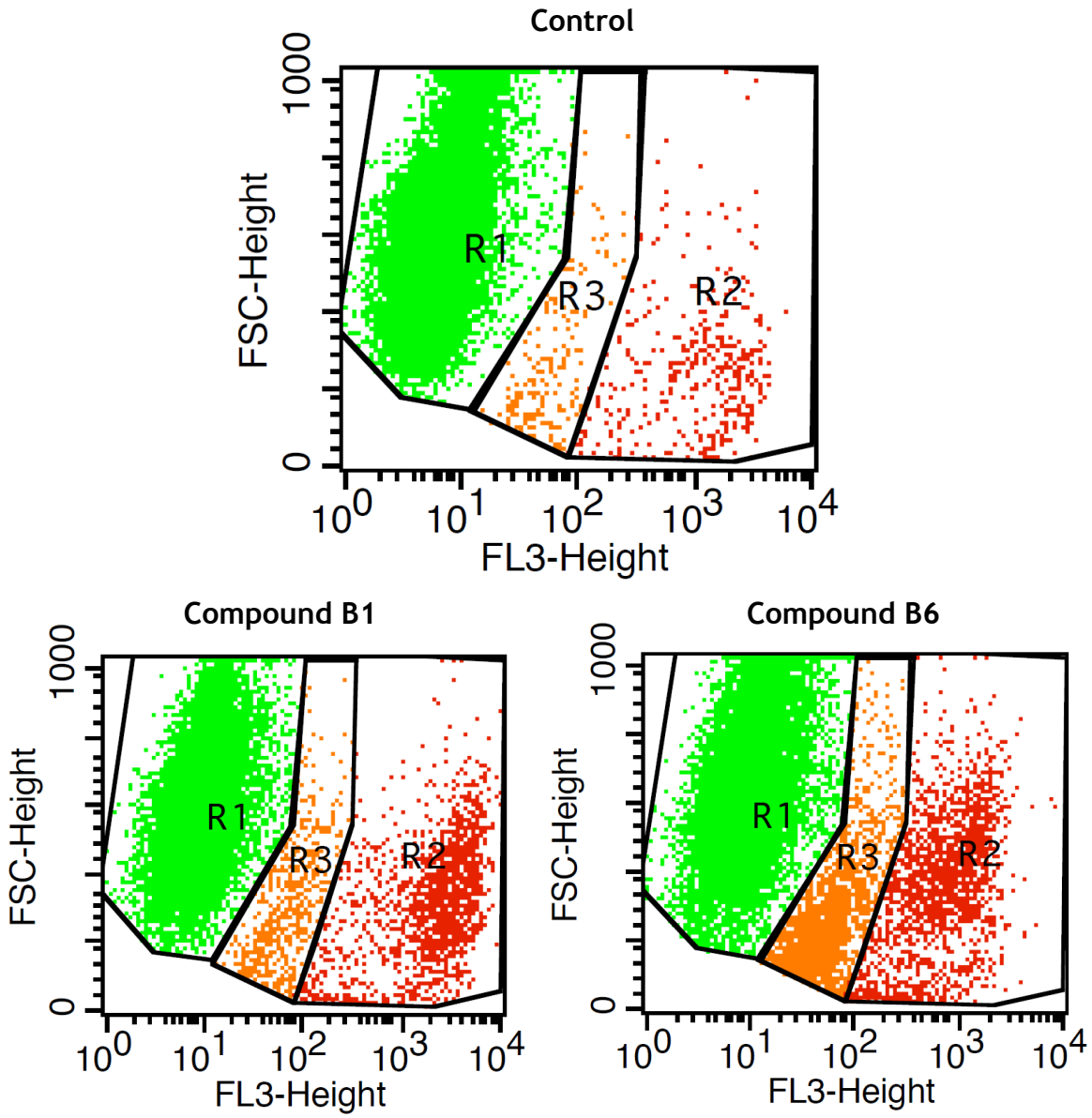


Figure 6 - Contour plots resulting of the analysis of the non-stained/stained cells with PI (size of events versus intensity of fluorescence) 24 hours after cell treatment; R1 - living cells; R2 - dead cells; R3 - intermediate population.

Analyzing figure 7, it is noteworthy that with both compounds there was a statistically significant decrease in the number of events at R1. It should also be noted that there was a statistically significant increase of dead cells, represented by the R2 region, with the exposure to both compounds B1 and B6. In the R3, intermediate region it can be seen a statistically significant increase of events with the exposure to compound B6. As referred previously, this can be due to early apoptotic or oncotic cells, cell debris, autoflourescent cells or the precipitated compound, that can be seen in figure 5(C). This data is congruent with the MTT assay data and the DCFDA assay data, in consideration of the diminution of the cell viability, and with the increased production of ROS, with compound B6. A hypothesis can be made here, perhaps the increase of ROS production lead to apoptosis, thus loss of cell viability, so in the flow cytometry studies a decrease, in relation to the control, of viable cells exposed to compound B6 (figure 7, R1) and an increase of the intermediate population (figure 7, R3) was observed.

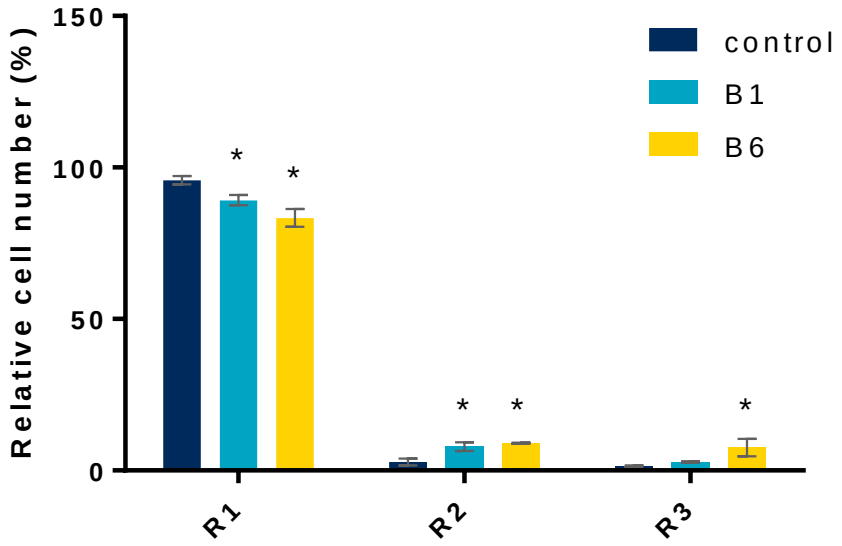


Figure 7 - Effects on cell viability at 24 hours of exposure to compounds B1 and B6 - results for the three regions (R1, R2 and R3). * statistically significant in relation to the control.

4.4 Thiols quantification

The DTND-thiols assay measures sulfhydryl groups with the thiol reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), which forms a mixed disulfide and the 5-thionitrobenzoic acid, a measurable yellow-colored product[119]. Sulfhydryl groups may be estimated in a sample by comparison to a standard curve composed of known concentrations of a sulfhydryl-containing compound, such as cysteine. The coupling of bismuth to organic compounds such as cysteine

can modify bismuth solubility and therefor it can perhaps also increase the toxicity of said compound[120].

First the cysteine calibration curve was determined, as can be seen in figure 8. Testing the two concentrations from each compound showed us that none of the compounds couple more than others to the thiols from cysteine (see table 5), which is rather strange, because we expected to see at least some differences between compounds. All the absorbances remain quite close to the one from the utilized cysteine concentration, which indicates, as stated above, that the compounds are not coupling with cysteine thiols in different degrees, in these experimental consitions, as would be expected.

We cannot achieve a conclusion in this case, since more studies should be done. A deduction could only be made if some bismuth compounds coupled with the cysteine thiols more than others, and only in that case could a relation be established between that coupling and the observed toxicities.

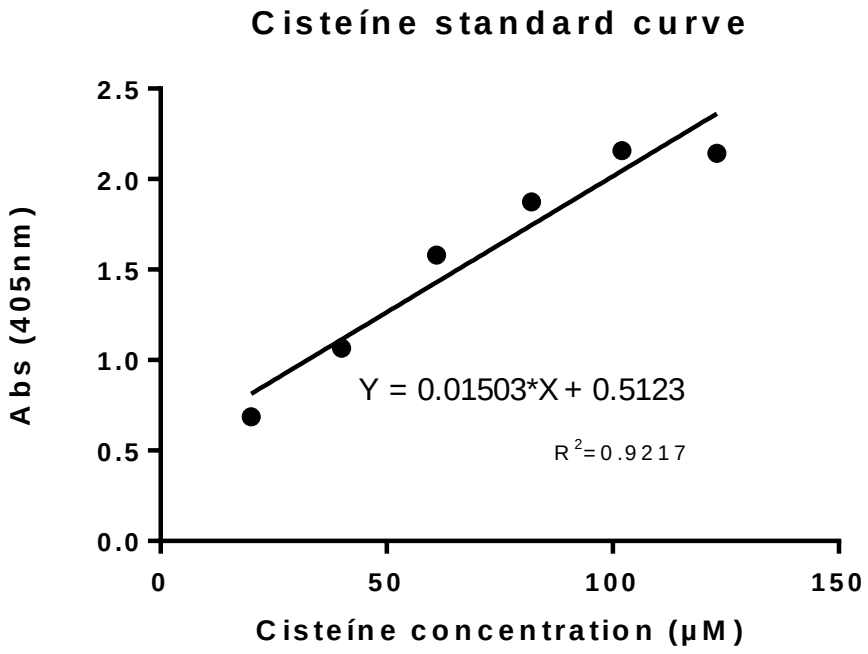


Figure 8 - Cysteine calibration curve

Table 5 - Absorbance at 405nm of all the compounds and cysteine

	Absorbance (405nm)	SD
Cysteine (100µM)	**2.048**	**0.052**
B1 82 µM	2.076	0.043
B1 123 µM	2.114	0.147
B2 82 µM	1.983	0.020
B2 123 µM	2.106	0.102
B3 82 µM	2.061	0.060
B3 123 µM	2.086	0.097
B4 82 µM	2.046	0.050
B4 123 µM	2.119	0.109
B5 82 µM	2.042	0.124
B5 123 µM	2.112	0.134
B6 82 µM	2.079	0.041
B6 123 µM	1.869	0.176
B7 82 µM	2.034	0.057
B7 123 µM	1.976	0.109
B8 82 µM	1.976	0.317
B8 123 µM	2.171	0.216
B9 82 µM	2.066	0.068
B9 123 µM	1.988	0.121
B10 82 µM	2.066	0.050
B10 123 µM	2.014	0.037

5 Conclusions and future work

In summary, cellular proliferation assays, the reactive oxygen species detection assays, and flow cytometry assays were all executed in order to evaluate the cytotoxicity of the ten bismuth compounds tested in this project and possibly the mechanism of the cytotoxicity. It can be concluded that most of the compounds tested do not exhibit a toxic profile in the cell cultures used for the experiments. But, the cellular cultures used in this study are only a small sample of the cell types that should be tested, particularly more studies should be made in the future with renal cells. Nonetheless, two compounds revealed a toxic profile in the N27 cell line and the Caco-2 cell line, showing that perhaps some more studies should be done, in order to fully comprehend the extent of the toxicity of bismuth compounds, as they do not seem to be as innocuous as they have been portrayed in the past. The two compounds with the most antiproliferative effects were B1 and B6, Bismuth(III) triflate and Bismuth(III) subnitrate, respectively; they were also tested by flow cytometry with propidium iodide staining, which showed some cellular death, which is congruent with the reactive oxygen species detection assay results, that showed some oxidative stress.

The study performed with the Ellman's reagent did not produce relevant results. With the 15-minute incubation period there seemed to not be any differences between the coupling of the different bismuth compounds to the cysteine thiols. So in the future this experiment should be repeated, perhaps with a longer incubation period or a different thiol source, to maybe correlate the possible toxicity of bismuth compounds to their augmented solubility by the coupling to thiols.

6 References

1. Mohan, R. Green bismuth. *Nat. Chem.* **2,** 336–336 (2010).
2. Suzuki, H. & Ogawa, T. Organobismuth(III) Compounds. *Organobismuth Chem*. 21–245 (2001).
3. Bismuth production top countries 2010-2015 | Statistic. *Statista* (2016). Available at: http://www.statista.com/statistics/264975/production-of-bismuth/. (Accessed: 12th September 2016)
4. Fowler, B. A., Sullivan Jr, D. W. & Sexton, M. J. *Chapter 31 - Bismuth. Handbook on the Toxicology of Metals (Fourth Edition)* **I,** (2015).
5. World bismuth reserves top countries 2015 | Statistic. *Statista* (2016). Available at: http://www.statista.com/statistics/264978/reserves-of-bismuth-by-major-countries/. (Accessed: 12th September 2016)
6. Woolrich, P. F. Occurence of Trace Metals in the Environment An Overview. *Am. Ind. Hyg. Assoc. J.* **34,** 217–226 (1973).
7. Filella, M. *et al.* How reliable are environmental data on 'orphan' elements? The case of bismuth concentrations in surface waters. *J. Environ. Monit.* **12,** 90–109 (2010).
8. Bowen, H. J. M. *Trace Elements in Biochemistry*. (Academic Press, 1966).
9. U.S. Food and Drug administration. Cosmetic Ingredient Listing. (Accessed: 13th September 2016)
10. Bradley, B., Singleton, M. & Po, A. L. W. Review Article Bismuth Toxicity-a Reassessment. *J. Clin. Pharm. Ther.* 423–441 (1989).
11. European Medicines Agency's Commitee on Medicinal Produtcs for Human Use. *List of nationally authorised medicinal products Active substance : cilostazol*. **44,** (2016).
12. Leonard, C. S. Studies in the pharmacology of bismuth salts. Tissue distribution of bismuth. *J. Pharmacol. Exp. Ther.* 333–345 (1928).
13. McCarthy, F. & Dexter, S. Oral manifestations of bismuth. *N. Engl. J. Med.* 345–353
14. Bismuth subsalicylate: Indications, Side Effects, Warnings - Drugs.com. Available at: https://www.drugs.com/cdi/bismuth-subsalicylate.html. (Accessed: 13th September 2016)
15. Chevretton, E. B., McRae, R. D. & Booth, J. B. Mastoidectomy packs: Xeroform or BIPP? *J. Laryngol. Otol.* **105,** 916–7 (1991).
16. Yang, N. & Sun, H. Biocoordination chemistry of bismuth: Recent advances. *Coord. Chem. Rev.* **251,** 2354–2366 (2007).
17. Ge, R. & Sun, H. Bioinorganic chemistry of bismuth and antimony: Target sites of metallodrugs. *Acc. Chem. Res.* **40,** 267–274 (2007).
18. N, Y. & Sun, H. *Biological Chemistry of Arsenic, Antimony and Bismuth*. *Biological chemistry of Arsenic, Antimony and Bismuth* (John Wiley & Sons Ltd, 2011).
19. Clinical Trials register - Search for bismuth. Available at: https://www.clinicaltrialsregister.eu/ctr-search/search?query=bismuth. (Accessed: 12th

September 2016)

20. Salvador, J. A., Figueiredo, S. A., Pinto, R. M. & Silvestre, S. M. Bismuth compounds in medicinal chemistry. *Future Med. Chem.* **4,** 1495–1523 (2012).

21. Gabbiani, C. *et al.* Outstanding plasmodicidal properties within a small panel of metallic compounds: Hints for the development of new metal-based antimalarials. *J. Inorg. Biochem.* **103,** 310–312 (2009).

22. Andrews, P. C. *et al.* Anti-Leishmanial activity of homo- and heteroleptic bismuth(III) carboxylates. *J. Inorg. Biochem.* **105,** 454–461 (2011).

23. Yang, N. *et al.* Bismuth complexes inhibit the SARS coronavirus. *Angew. Chemie - Int. Ed.* **46,** 6464–6468 (2007).

24. Li, H. & Sun, H. Recent advances in bioinorganic chemistry of bismuth. *Curr. Opin. Chem. Biol.* **16,** 74–83 (2012).

25. Silvestru, C., Breunig, H. J. & Althaus, H. Structural Chemistry of Bismuth Compounds. I. Organobismuth Derivatives. *Chem. Rev.* **99,** 3277–3327 (1999).

26. Li, W. *et al.* Structure of colloidal bismuth subcitrate (CBS) in dilute HCl: Unique assembly of bismuth citrate dinuclear units ([Bi(cit)2Bi] 2-). *J. Am. Chem. Soc.* **125,** 12408–12409 (2003).

27. Andrews, P. C. *et al.* Towards a structural understanding of the anti-ulcer and anti-gastritis drug bismuth subsalicylate. *Angew. Chemie - Int. Ed.* **45,** 5638–5642 (2006).

28. Chey, W. D., Wong, B. C. Y. & Practice Parameters Committee of the American College of Gastroenterology. American College of Gastroenterology Guideline on the Management of *Helicobacter pylori* Infection. *Am. J. Gastroenterol.* **102,** 1808–1825 (2007).

29. Malfertheiner, P. *et al.* Current concepts in the management of *Helicobacter pylori* infection: The Maastricht III Consensus Report. *Gut* **56,** 772–81 (2007).

30. Malfertheiner, P. *et al. Helicobacter pylori* eradication with a capsule containing bismuth subcitrate potassium, metronidazole, and tetracycline given with omeprazole versus clarithromycin-based triple therapy: A randomised, open-label, non-inferiority, phase 3 trial. *Lancet* **377,** 905–913 (2011).

31. Tsang, C. N., Ho, K. S., Sun, H. & Chan, W. T. Tracking bismuth antiulcer drug uptake in single *helicobacter pylori* cells. *J. Am. Chem. Soc.* **133,** 7355–7357 (2011).

32. Ge, R. *et al.* A proteomic approach for the identification of bismuth-binding proteins in Helicobacter pylori. *J. Biol. Inorg. Chem.* **12,** 831–842 (2007).

33. Cun, S. & Sun, H. A zinc-binding site by negative selection induces metallodrug susceptibility in an essential chaperonin. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 4943–4948 (2010).

34. Chen, R. *et al.* Fabrication of bismuth subcarbonate nanotube arrays from bismuth citrate. *Chem. Commun. (Camb).* 2265–7 (2006). doi:10.1039/b601764a

35. Chen, R. *et al.* Bismuth subcarbonate nanoparticles fabricated by water-in-oil microemulsion-assisted hydrothermal process exhibit anti-*Helicobacter pylor*i properties. *Mater. Res. Bull.* **45,** 654–658 (2010).

36. Shaikh, A. R., Giridhar, R., Megraud, F. & Yadav, M. R. Metalloantibiotics: synthesis,

characterization and antimicrobial evaluation of bismuth-fluoroquinolone complexes against *Helicobacter pylori*. *Acta Pharm.* **59,** 259–271 (2009).

37. Andrews, P. C. *et al*. Bismuth(III) complexes derived from non-steroidal anti-inflammatory drugs and their activity against *Helicobacter pylori*. *Dalton Trans.* **39,** 2861–8 (2010).

38. Andrews, P. C. *et al*. Bismuth(III) 5-sulfosalicylate complexes: structure, solubility and activity against *Helicobacter pylori*. *Dalton Trans.* **28,** 6377–84 (2009).

39. Andrews, P. C. *et al*. Structural and solution studies of phenylbismuth(III) sulfonate complexes and their activity against Helicobacter pylori. *Dalton Trans.* **39,** 9633–41 (2010).

40. Kotani, T. *et al*. Antibacterial Properties of Some Cyclic Organobismuth (III) Compounds. *Antimicrob. Agents Chemother.* **49,** 2729–2734 (2005).

41. Chauhan, H. P. S., Shaik, N. M. & Singh, U. P. Synthetic, spectroscopic and antimicrobial studies of bis(dialkyldithiocarbamato)diorganodithiophosphatobismuth(III) complexes. *Appl. Organomet. Chem.* **19,** 1132–1139 (2005).

42. Chauhan, H. P. S., Bakshi, A. & Bhatiya, S. Synthetic, spectral as well as *in vitro* antimicrobial studies on some bismuth(III) bis (N , N -dialkyldithiocarbamato) alkylenedithiophosphates. *Appl. Organomet. Chem.* **24,** 317–325 (2010).

43. Tripathi, U. N., Siddiqui, A. & Solanki, J. S. Synthesis , Spectral Characterization , and Antimicrobial Activity of Arsenic (III) and Bismuth (III) phenyl) pyrazolinates]. **33,** 257–266 (2009).

44. Solanki, J. S., Thapak, T. R. & Bhardwaj, A. Synthesis , structural characterization , and in vitro antimicrobial properties of salicylate and pyrazoline complexes of bismuth (III). *J. Coord. Chem.* **64,** 369–376 (2011).

45. Dawara, L. & Singh, R. V. Microwave-assisted synthesis, characterization, antimicrobial, and pesticidal activity of bismuth and antimony complexes with coumarin-based ligands. *J. Coord. Chem.* **64,** 931–941 (2011).

46. Shaikh, A. R., Giridhar, R. & Yadav, M. R. Bismuth-norfloxacin complex: Synthesis, physicochemical and antimicrobial evaluation. *Int. J. Pharm.* **332,** 24–30 (2007).

47. Halwani, M. *et al*. Liposomal bismuth-ethanedithiol formulation enhances antimicrobial activity of tobramycin. *Int. J. Pharm.* **358,** 278–284 (2008).

48. Alipour, M., Suntres, Z. E., Lafrenie, R. M. & Omri, A. Attenuation of Pseudomonas aeruginosa virulence factors and biofilms by co-encapsulation of bismuth-ethanedithiol with tobramycin in liposomes. *J. Antimicrob. Chemother.* **65,** 684–693 (2010).

49. Halwani, M. *et al*. Bismuth-thiol incorporation enhances biological activities of liposomal tobramycin against bacterial biofilm and quorum sensing molecules production by *Pseudomonas aeruginosa*. *Int. J. Pharm.* **373,** 141–146 (2009).

50. Murafuji, T. *et al*. Antifungal activity of organobismuth compounds against the yeast *Saccharomyces cerevisiae*: Structure-activity relationship. *J. Inorg. Biochem.* **98,** 547–552 (2004).

51. Murafuji, T. *et al*. Bismuth heterocycles based on a diphenyl sulfone scaffold: Synthesis and substituent effect on the antifungal activity against *Saccharomyces cerevisiae*. *Eur. J. Med. Chem.* **46,** 519–525 (2011).

52. Sousa, M. C. & Poiares-Da-Silva, J. Cytotoxicity induced by bismuth subcitrate in Giardia lamblia trophozoites. *Toxicol. Vitr.* **13,** 591–598 (1999).

53. Kim, Y. S. & Brechbiel, M. W. An overview of targeted alpha therapy. *Tumor Biol.* **33,** 573–590 (2012).

54. Brechbiel, M. W. Targeted α-therapy: past, present, future? *Dalt. Trans.* 4918 (2007).

55. Bierer, D. Bismuth subsalycilate. History, chemistry, and safety. *Rev Infect Dis* **12,** 3–8 (1990).

56. Treiber, G., Gladziwa, U. & Ittel, T. Tripotassium dicitrato bismuthate: absorption and urinary excretion of bismuth in patients with normal and impaired renal function. *Pharmacol Ther* 491–502 (1991).

57. Lacey, L., Frazer, N., Keene, O. & Smith, J. Comparative pharmacokinetics of bismuth from ranitidine bismuth citrate (GR122311X), a novel anti-ulcerant, and tri-potassium dicitrato bismuthate (TDB). *Eur J Clin Pharmacol* 177–180 (1994).

58. Slikkerveer, A. & de Wolff, F. A. Pharmacokinetics and toxicity of bismuth compounds. *Med. Toxicol. Adverse Drug Exp.* **4,** 303–23

59. Larsen, A., Martiny, N., Stoltenberg, M., Danscher, G. & Rungby, J. Gastrointestinal and Systemic Uptake of Bismuth in Mice after Oral Exposure. *Pharmacol. Toxicol.* **93,** 82–90 (2003).

60. Von Recklinghausen, U. *et al.* Methylated bismuth, but not bismuth citrate or bismuth glutathione, induces cyto- and genotoxic effects in human cells in vitro. *Chem. Res. Toxicol.* **21,** 1219–1228 (2008).

61. Russ, G., Bigler, R., Tilbury, R., Woodard, H. & Laughlin, J. Metabolic studies with radiobismuth, I: retention and distribution of 206Bi in the normal rat. *Radiat. Res.* 443–454 (1975).

62. Thomas, D. W., Sobecki, S., Hartley, T. F., Coyle, P. & Alp, M. H. *Chemical toxicology and clinical chemistry of metals.* (Academic Press, 1983).

63. Sollmann, T., Seifter, J., Mangun, G. H., Ramvouzec, E. S. & O'Hare, J. A. Intravenous injections of soluble bismuth compounds: their toxicity, and their sojourn in the blood and organs. *J. Pharmacol. Exp. Ther.* **74,** 134–154 (1942).

64. Lee, S. P., Lim, T. H., Pybus, J. & Clarke, A. C. Tissue distribution of arally administred bismuth in the rat. *Clin. Exp. Pharmacol. Physiol.* **7,** 319–324 (1980).

65. Escourolle, R., Bourdon, R., Galli, A., Galle, P. & Jaudon, M. C. Étude neuropathologique et toxicologique de douze cas d'encéphalopathie bismuthique. *Rev. Neurol. (Paris).* 153–163 (1977).

66. Kondo, Y., Satoh, M., Imura, N. & Akimoto, M. Effect of bismuth nitrate given in combination with cis-diamminedichloroplatinum(II) on the antitumor activity and renal toxicity of the latter in nude mice inoculated with human bladder tumor. *Cancer Chemother. Pharmacol.* **29,** 19–23 (1991).

67. Kondo, Y., Satoh, M., Imura, N. & Akimoto, M. Tissue-specific induction of metallothionein by bismuth as a promising protocol for chemotherapy with repeated administration of cis-diamminedichloroplatinum (II) against bladder tumor. *Anticancer Res.* **12,** 2303–7

68. Kondo, Y. *et al.* Citrate enhances the protective effect of orally administered bismuth subnitrate against the nephrotoxicity of cis-diamminedichloroplatinum. *Cancer Chemother. Pharmacol.* **53,** 33–8 (2004).

69. Satoh, M., Naganuma, A. & Imura, N. Modulation of adriamycin toxicity by tissue-specific induction of metallothionein synthesis in mice. *Life Sci.* **67,** 627–34 (2000).

70. Naruse, I. & Hayashi, Y. Amelioration of the teratogenicity of cadmium by the metallothionein induced by bismuth nitrate. *Teratology* **40,** 459–65 (1989).

71. Satoh, M. *et al.* Prevention of adverse effects of gamma-ray irradiation after metallothionein induction by bismuth subnitrate in mice. *Eur. J. Cancer Clin. Oncol.* **25,** 1727–31 (1989).

72. Boertz, J. *et al.* Determination of Trimethylbismuth in the Human Body after Ingestion of Colloidal Bismuth Subcitrate. *Drug Metab. Dispos.* **37,** 352–358 (2009).

73. Hollmann, M., Boertz, J., Dopp, E., Hippler, J. & Hirner, A. V. Parallel on-line detection of a methylbismuth species by hyphenated GC/EI-MS/ICP-MS technique as evidence for bismuth methylation by human hepatic cells. *Metallomics* **2,** 52–6 (2010).

74. Soutar, R. L. *et al.* Interaction of tripotassium dicitrato bismuthate with macrophages in the rat and in vitro. *Gastroenterology* **91,** 84–93 (1977).

75. Boudène, C., Bouley, G., Belegaud, J. & Comoy, E. Recherches étiologiques et pathogéniques expérimentales sur la toxicité du sousnitrate de bismuth lourd micronisé chez le rat. *J. Pharmacol.* 93–104 (1979).

76. Sollmann, T. & Seifter, J. The pharmacology of trimethyl bismuth. *J. Pharmacol. Exp. Ther.* **67,** 17–49 (1939).

77. Benet, L. Safety and pharmacokinetics: colloidal bismuth subcitrate. *Scand J Gastroenterol* **26,** 29–35 (1991).

78. Leonard, C. S. & Love, R. B. Studies in the pharmacology of bismuth salts VI. The permeability of the placenta to bismuth. *J. Pharmacol. Exp. Ther.* **34,** 347–354 (1928).

79. Supino-Viterbo, V., Sicard, C., Risvegliato, M., Rancurel, G. & Buge, a. Toxic encephalopathy due to ingestion of bismuth salts: clinical and EEG studies of 45 patients. *J. Neurol. Neurosurg. Psychiatry* **40,** 748–752 (1977).

80. Stoltenberg, M. & Danscher, G. Histochemical differentiation of autometallographically traceable metals (Au, Ag, Hg, Bi, Zn): Protocols for chemical removal of separate autometallographic metal clusters in Epon sections. *Histochem. J.* **32,** 645–652 (2000).

81. Pamphlett, R., Danscher, G., Rungby, J. & Stoltenberg, M. Tissue uptake of bismuth from shotgun pellets. *Environ. Res.* **82,** 258–262 (2000).

82. Stoltenberg, M., Schiønning, J. D. & Danscher, G. Retrograde axonal transport of bismuth: an autometallographic study. *Acta Neuropathol.* **101,** 49–52 (2001).

83. Stoltenberg, M., Danscher, G., Pamphlett, R., Christensen, M. M. & Rungby, J. Histochemical tracing of bismuth in testis from rats exposed intraperitoneally to bismuth subnitrate. *Reprod. Toxicol.* **14,** 65–71 (2000).

84. Stoltenberg, M., Larsen, a, Zhao, M., Danscher, G. & Brunk, U. T. Bismuth-induced lysosomal rupture in J774 cells. *APMIS* **110,** 396–402 (2002).

85. Işlek, I., Uysal, S., Gök, F., Dündaröz, R. & Küçüködük, Ş. ¸. Reversible nephrotoxicity after overdose of colloidal bismuth subcitrate. *Pediatr. Nephrol.* **16,** 510–514 (2001).

86. Cengiz, N., Uslu, Y., Gök, F. & Anarat, A. Acute renal failure after overdose of colloidal bismuth subcitrate. *Pediatr. Nephrol.* **20,** 1355–1358 (2005).

87. Erden, A. *et al.* A case of bismuth intoxication with irreversible renal damage. *Int. J. Nephrol. Renovasc. Dis.* **6,** 241–243 (2013).

88. Dopp, E. *et al.* Toxicity of volatile methylated species of bismuth, arsenic, tin, and mercury in Mammalian cells in vitro. *J. Toxicol.* **2011,** 503576 (2011).

89. Luo, Y. *et al.* In vitro cytotoxicity of surface modified bismuth nanoparticles. *J. Mater. Sci. Mater. Med.* **23,** 2563–2573 (2012).

90. Song, Q. *et al.* The acute cytotoxicity of bismuth ferrite nanoparticles on PC12 cells. *J. Nanoparticle Res.* **16,** 2408 (2014).

91. Gao, X. *et al.* Comparative toxicities of bismuth oxybromide and titanium dioxide exposure on human skin keratinocyte cells. *Chemosphere* **135,** 83–93 (2015).

92. Ano, Y. S. *et al.* Oral Toxicity of Bismuth in Rat : Single and 28-Day Repeated Administration Studies. 293–298 (2005).

93. Ano, Y. S. *et al.* A 13-Week Toxicity Study of Bismuth in Rats by Intratracheal Intermittent Administration. 242–248 (2005).

94. Kruger, G., Thomas, D. J., Weinhardt, F. & Hoyer, S. Disturbed Oxidative Metabolism in Organic Brain Syndrome Caused By Bismuth in Skin Creams. *Lancet* 485–487 (1976).

95. Hudson, M. & Mowat, N. a. Reversible toxicity in poisoning with colloidal bismuth subcitrate. *BMJ* **299,** 159 (1989).

96. Taylor, E. G. & Klenerman, P. Acute renal failure after colloidal bismuth subcitrate overdose. *Lancet* 670–671 (1990).

97. R.J;, P. *et al.* Bismuth induced encephalopathy caused by tri potassium dicitrato bismuthate in a patient with chronic renal failure. *Gut* **31,** 359–360 (1990).

98. Huwez, F., Pall, A., Lyons, D. & Stewart, M. J. Acute renal failure after overdose of colloidal bismuth subcitrate. *Lancet* 1298 (1992).

99. Akpolat, I. *et al.* Acute renal failure due to overdose of colloidal bismuth. *Nephrol. Dial. ransplantationransplantation* 1890,1891 (1996).

100. Summers, W. K. Bismuth Toxicity Masquerading as Alzheimer's Dementia. *J. Alzheimers. Dis.* **1,** 57–59 (1998).

101. Hruz, P., Mayr, M., Löw, R., Drewe, J. & Huber, G. Fanconi's syndrome, acute renal failure, and tonsil ulcerations after colloidal bismuth subcitrate intoxication. *Am. J. Kidney Dis.* **39,** 1–5 (2002).

102. Reynolds, P. T. Bismuth Toxicity: A Rare Cause of Neurologic Dysfunction. *Int. J. Clin. Med.* **3,** 46–48 (2012).

103. Akinci, E. *et al.* Acute bismuth intoxication: Acute renal failure, tonsillar ulceration and posterior reversible encephalopathy syndrome. *Hong Kong J. Emerg. Med.* **22,** 121–125 (2015).

104. Sharma, R. R., Cast, I. P., Redfern, R. M. & O'Brien, C. Extradural application of bismuth iodoform paraffin paste causing relapsing bismuth encephalopathy: a case report with CT and MRI studies.

J. Neurol. Neurosurg. Psychiatry **57,** 990–3 (1994).

105. Harris, R. A. & Poole, A. Beware of bismuth: Post maxillectomy delirium. *ANZ J. Surg.* **72,** 846–847 (2002).

106. MA, R., S, S. & DI, O. Allergic contact otitis externa due to iodoform in BIPP cavity dressings. *Contact Dermatitis* **46,** 360 (2002).

107. Youngman, L. & Harris, S. BIPP madness; an iatrogenic cause of acute confusion. *Age Ageing* **33,** 406–407 (2004).

108. Ovaska, H. *et al.* Severe iatrogenic bismuth poisoning with bismuth iodoform paraffin paste treated with DMPS chelation. *Clin. Toxicol.* **46,** 855–857 (2008).

109. Atwal, a & Cousin, G. C. S. Bismuth toxicity in patients treated with bismuth iodoform paraffin packs. *Br. J. Oral Maxillofac. Surg.* **54,** 111–2 (2016).

110. Thomas, F., Bialek, B. & Hensel, R. Medical Use of Bismuth : the Two Sides of the Coin. *J. Clin. Toxicol.* **2012,** S3-4 (2012).

111. Wu, D. & Yotnda, P. Production and detection of reactive oxygen species (ROS) in cancers. *J. Vis. Exp.* (2011). doi:10.3791/3357

112. Riss, T. L. *et al. Cell Viability Assays. Assay Guidance Manual* (Eli Lilly & Company and the National Center for Advancing Translational Sciences, 2004).

113. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65,** 55–63 (1983).

114. Wojtala, A. *et al.* in *Methods in Enzymology* **542,** 243–262 (2014).

115. Roesslein, M., Hirsch, C., Kaiser, J.-P., Krug, H. F. & Wick, P. Comparability of in vitro tests for bioactive nanoparticles: a common assay to detect reactive oxygen species as an example. *Int. J. Mol. Sci.* **14,** 24320–37 (2013).

116. Liou, G.-Y. & Storz, P. Reactive oxygen species in cancer. *Free Radic. Res.* **44,** 479–96 (2010).

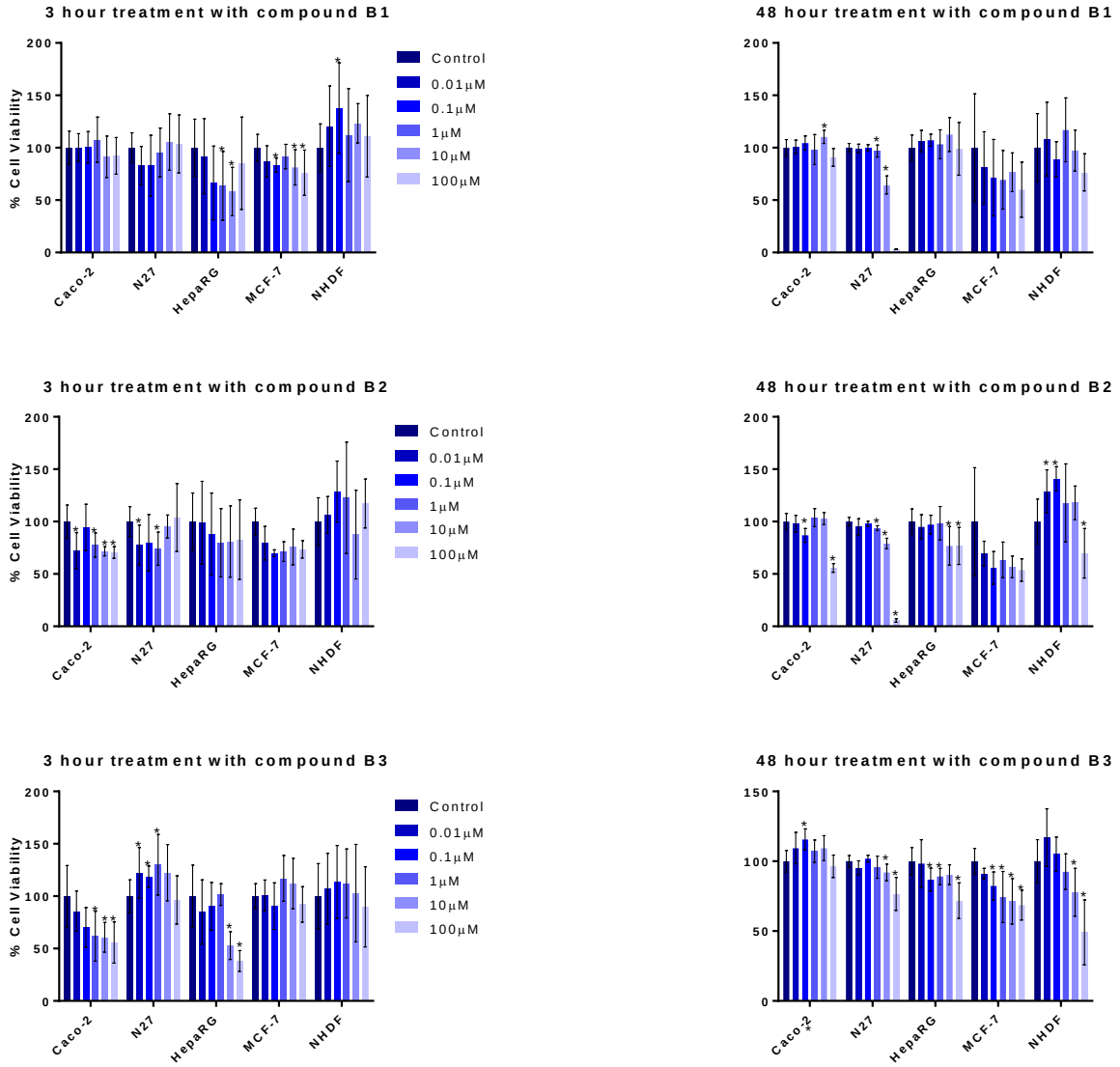
117. Circu, M. L. & Aw, T. Y. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic. Biol. Med.* **48,** 749–62 (2010).

118. Henry, C. M., Hollville, E. & Martin, S. J. Measuring apoptosis by microscopy and flow cytometry. *Methods* **61,** 90–7 (2013).

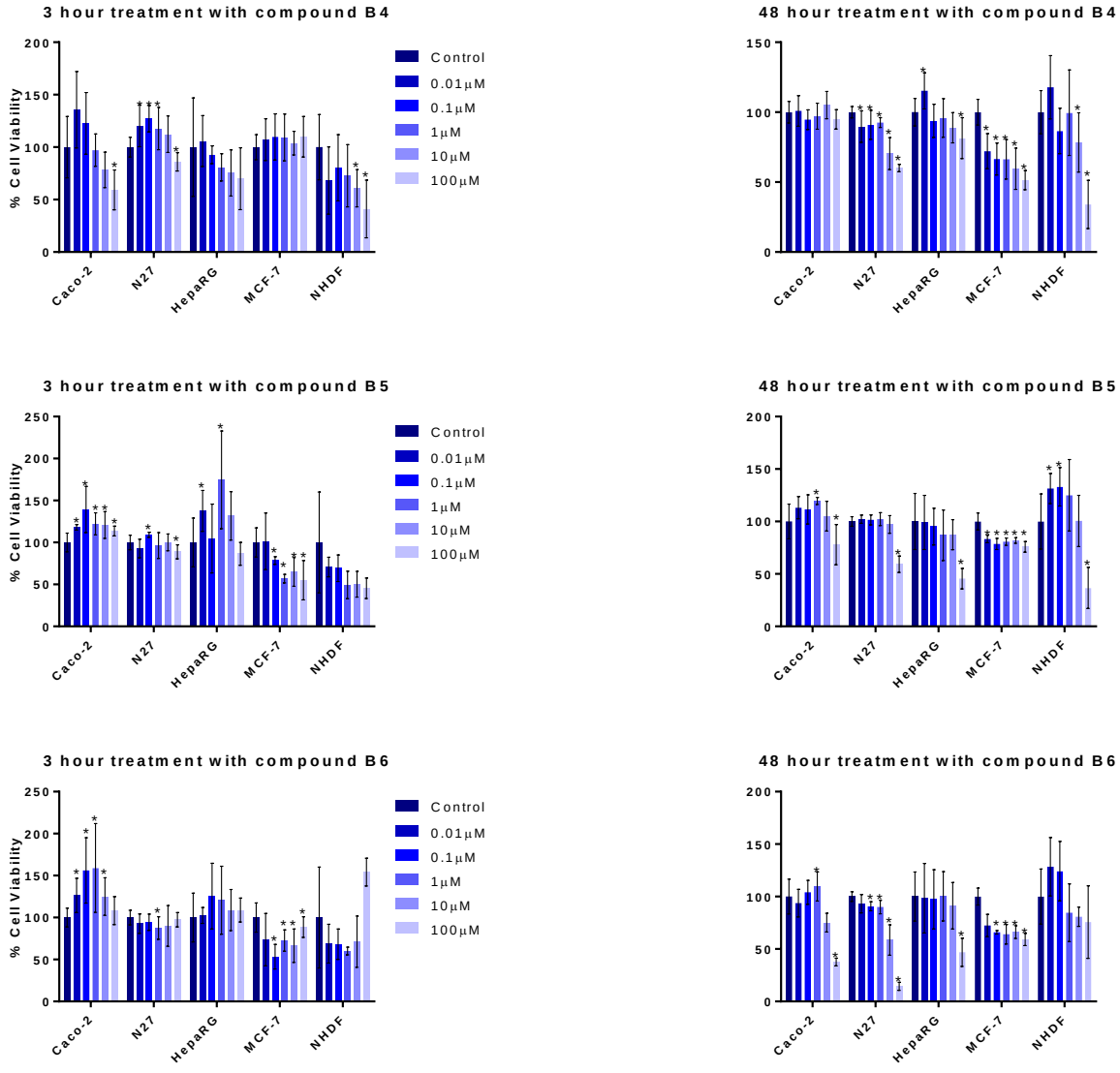
119. Ellman, G. L., Courtney, K. D., Andres, V. & Featherstone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7,** 88–95 (1961).

120. Krari, N., Mauras, Y. & Allain. Enhancement of bismuth toxicity by l-cysteine. *Res. Commun. Mol. Pathol. Pharmacol.* **89,** 357–64 (1995).

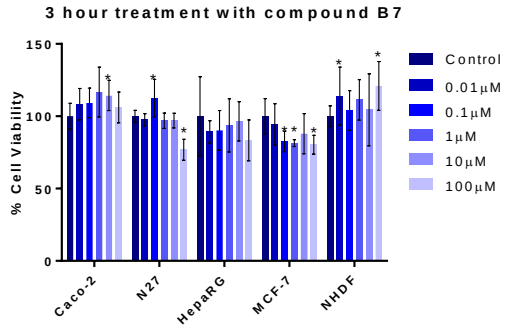
7 Attachments

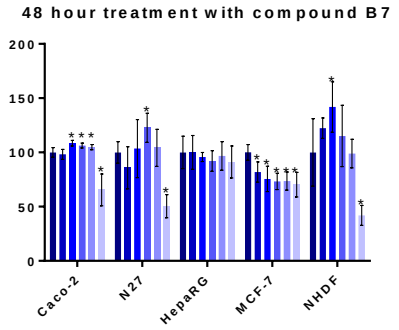


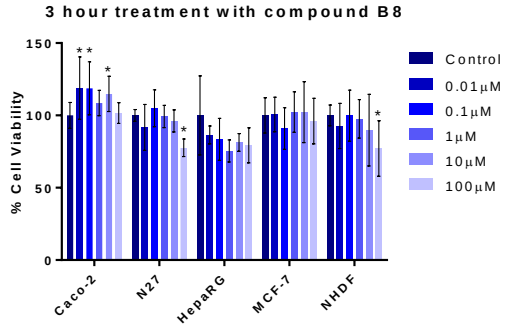
Attachment 1 - Relative proliferation of Caco-2, N27, HepaRG, MCF-7 and NHDF cell lines after 3 and 48h exposure to compounds 1, 2, and 3 (results expressed as mean ± SD) * $p<0.05$ in relation to the control (t-student test).

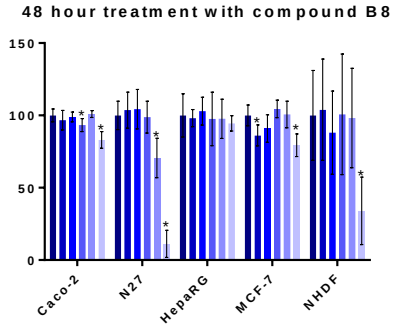


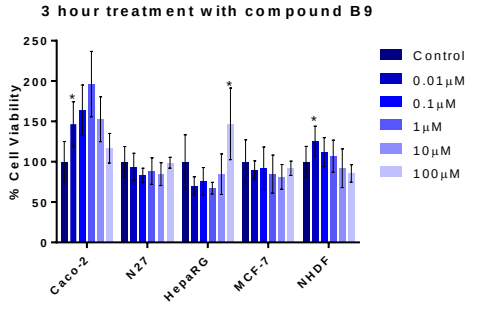
Attachment 2 - Relative proliferation of Caco-2, N27, HepaRG, MCF-7 and NHDF cell lines after 3 and 48h exposure to compounds 4,5 and 6 (results expressed as mean ± SD) * $p<0.05$ in relation to the control (t-student test).

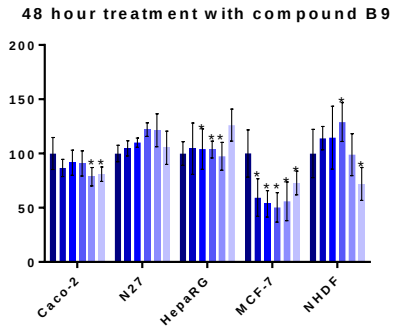


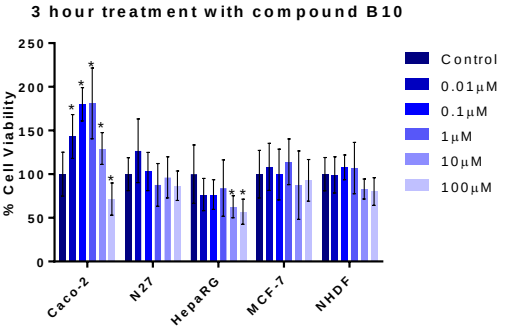


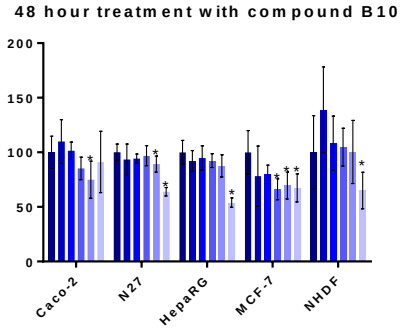












Attachment 2 - Relative proliferation of Caco-2, N27, HepaRG, MCF-7 and NHDF cell lines after 3 and 48h exposure to compounds 7, 8, 9 and 10 (results expressed as mean ± SD) * $p<0.05$ in relation to the control (t-student test).